ANTIOXIDANT AND ANTIGLYCATION EFFECT OF SOME PHYTOCHEMICALS

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

SHAZIA AMAN

Dated : .....

Approved : .....

.....

.....

Dr. Shagufta Moin (Supervisor)

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ALIGARH (INDIA)

2013

Dedicated

To

My Parents

DEPARTMENT OF BIOCHEMISTRY
Faculty of Medicine
Jawaharlal Nehru Medical College
Aligarh Muslim University, Aligarh-202002 (INDIA)

Certificate

This is to certify that the thesis entitled *"Antioxidant and Antiglycation effect of some Phytochemicals"* ***herewith submitted by*** *Shazia Aman,* ***in fulfillment of the requirements for the degree of*** *Doctor of Philosophy in Biochemistry* ***of the Aligarh Muslim University, is an authentic record of the research work carried out by her under our supervision and guidance and that no part, thereof, has been presented before for any other degree.***

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Dated: April, 2013

ETHICAL CLEARANCE CERTIFICATE

Subject: Reference research work submitted by Dr. Shazia Aman, Research Scholar (Ph.D), Department of Biochemistry.

The Institutional Ethics Committee, Faculty of Medicine AMU, Aligarh has reviewed and discussed your research work on the topic **"Antioxidant and Antiglycation effect of some Phytochemicals".**

After consideration, the committee has decided to approve the study under the referenced topic subject to the following conditions:

- It is understood that the study is being conducted at J.N. Medical College & Hospital, AMU, Aligarh.
- Any serious adverse event that occurs during the conduct of the study at J.N. Medical College & Hospital, AMU, Aligarh, should be reported to the Ethics Committee immediately for review if required.
- The Study will be conducted after informed consent from patient/guardian.

22/4/2013
(Prof. M.H. Beg)
DEAN
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To,

Dr. Shazia Aman, D/o Biochemistry,
Research Scholar (Ph.D),
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Shazia

(SHAZIA AMAN)

CONTENTS

A	Absorbance
AA	Ascorbic acid
AAPH	2,2-azobis (2-methylpropionamidine) dihydrochloride
AG	Aminoguanidine
AGEs	Advanced glycation end products
BMI	Basal metabolic Index
BSA	Bovine Serum Albumin
CD	Circular Dichroism
Cl^-	Chloride ion
cm	centimetre
Cu^+	Cuprous ion
Cu^{2+}	Cupric ion
CVD	Cardiovascular disease
dl	decilitre
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DNPH	2,4- Dinitrophenyl hydrazine
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTNB	5,5'- dithiobis-2-nitrobenzoic acid
DTPA	Diethylene triamine penta-acetic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EU	Eugenol
EU-1	Eugenol at 0.06 μM concentration
EU-2	Eugenol at 0.6 μM concentration
EU-3	Eugenol at 6.0 μM concentration
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
$FeCl_2$	Ferrous chloride

$FeCl_3$	Ferric chloride
$FeSO_4.7H_2O$	Ferrous sulphate
FI	Fluorescence Intensity
FRAP	Ferric Reducing Antioxidant Potential
FTIR	Fourier Transform Infrared Spectroscopy
g	gram
GA	Gallic acid
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
H	Native HSA
H^+	Hydrogen ion
HbA_1c	Glycated Haemoglobin
HCl	Hydrochloric Acid
HG	HSA + Glucose
HGA	HSA + Glucose + Aminoguanidine
H_2O_2	Hydrogen Peroxide
HOCl	Hypochlorous acid
HSA	Human Serum Albumin
kD	kilodalton
$[K_3Fe(CN)_6]$	Potssium Ferricyanide
L	Litre
LDL	Low Density Lipoprotein
M	Molarity
MDA	Malondialdehyde
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MRE	Mean Residue Ellipticity

N	Normality
$NADP^+$	Nicotinamide Adenine Dinucleotide (oxidised)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NaOH	Sodium Hydroxide
NBT	Nitroblue Tetrazolium Chloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometre
nmoles	nanomoles
$NO^{\bullet}$	Nitric Oxide radical
NO_2	Nitrogen Dioxide
NO_3	Nitrate
O_2	Oxygen molecule
$O_2^{\bullet-}$	Superoxide ion
$OH^{\bullet}$	Hydroxyl radical
$ONOOCO_2^-$	Nitroso Peroxocarboxylate
ONOOH	Peroxonitrous acid
$OONO^-$	Peroxynitrite
OPD	Out Patient Department
p	Probability of error
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
RAGE	Receptors for Advanced Glycation End Products
RBC	Red Blood Cells
RNA	Ribonucleic Acid
$RO^{\bullet}$	Alkoxyl radical
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive Oxygen Species
rpm	revolutions per minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate

—SH	Sulfhydryl group
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TEMED	N,N,N,N-tetramethyethylene diamine
TL	Thymol
TL-1	Thymol at 3 µM concentration
TL-2	Thymol at 30 µM concentration
TL-3	Thymol at 300 µM concentration
TNBS	2,4,6- trinitrobenzene sulphonic acid
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
TQ	Thymoquinone
TQ-1	Thymoquinone at 3 µM concentration
TQ-2	Thymoquinone at 30 µM concentration
TQ-3	Thymoquinone at 300 µM concentration
UV	Ultraviolet
Zn^{2+}	Zinc ion
α	alpha
β	beta
ε	epsilon
µg	microgram
µl	microliter
µM	micromolar

Natural antioxidants from plant products have been reported to possess free radical scavenging properties and prevent oxidative damage without causing unwanted effects. Among various classes of chemicals present in the plants, phenolic compounds have tremendous health promoting effects as antioxidants. The phenolic compounds exhibit considerable free radical scavenging activities through their electron-donating and metal ion chelating properties. Since some antioxidants may act as inhibitors of glycation, in the first phase of study, we evaluated in-vitro antioxidant activities of three phytochemicals: thymoquinone (TQ), thymol (TL) and eugenol (EU) by determining reducing power, ferric reducing antioxidant power (FRAP), metal chelating activity, DPPH and AAPH radical scavenging assays. The phytochemicals were also found to have properties of chelating ferrous ions. Based on these data, it is clear that the TQ, TL and EU contained high antioxidant activity and chelating properties. The all three phytochemicals were found to possess strong radical-scavenging and redox abilities as evidenced by model antioxidant assays including DPPH and FRAP. To evaluate whether the tested phytochemicals could affect glycation induced generation of ROS, cytochrome c reduction assay was employed. A significant decrease in the generation of superoxide radical was observed in the presence of these phytochemicals. This seemingly indicates that the phytochemicals either scavenge superoxide anion radicals or chelate transition metals leading to less free radical production, or they may have both effects. The protective effect of these phytochemicals on AAPH-induced RBC hemolysis was observed in a dose dependent manner. EU possesses lower IC_{50} value as compared to TL and TQ as shown by higher inhibition of RBC hemolysis. Similarly, reducing power and FRAP value was found to be higher in case of EU as compared to TL and TQ. The order of antioxidant activity of these compounds was same in all antioxidant parameters tested viz. TQ<TL<EU. Among three phytochemicals, EU had the most powerful antioxidant and radical scavenging activity followed by TL and TQ which is related to their structure. TQ has weak electron donating groups contributing to less electron density to the benzene ring. However, electron density is found to be higher in TL and EU because of presence of hydroxyl group attached to benzene ring. Among EU and TL, EU has two electron donating groups viz. methoxy group (—OCH_3) and allyl group (—CH_2—$CH{=}CH_2$) at ortho- and para- position respectively which are more reactive sites as compared to meta- position while TL has weak electron donating groups at ortho- and meta- position (methyl and isopropyl) respectively. Hence it can be concluded that EU having maximum electron density showed best antioxidant activity among the three phytochemicals.

Non-enzymatic glycation, the reaction of glucose and other reducing sugars with amino group of proteins, produces Amadori or early glycation products, while longer exposure results in irreversible advanced glycation end products (AGEs). Glycation involves non-enzymatic covalent attachment of carbonyl groups of glucose with N-terminal and lysyl side chain ε-amino groups to form unstable Schiff base adduct that rapidly progresses to a

stable ketoamine derivative, the Amadori product. Hereafter, the reactions become more varied and complicated leading to the formation of AGEs. AGE formation is greatly accelerated in hyperglycaemic conditions and many studies so far have demonstrated the formation and role of AGEs in various diseases including diabetes. Glycation alters protein conformation and induces protein cross linking that eventually ensues in aggregation. Modifications of structural as well as circulatory proteins by glycation have drawn much attention because of their potential role in the etiopathogenesis of various diseases.

As the glycation is a dose and time dependent process, in the second phase, a comparative study of HSA denaturation/degradation induced by glucose for extended time period was performed. The structural perturbations in the glycated HSA samples were analyzed by UV absorbance, tryptophan fluorescence, circular dichroism, FTIR and gel electrophoresis techniques. The studies revealed remarkable structural and biophysical changes in HSA upon glycation by glucose up to 28 days. Estimation of ketoamine, carbonyl and free amino groups revealed that glycation induces conformational and structural changes in HSA. UV spectra of glycated HSA showed hyperchromicity as observed on day 14 and post incubation hypochromicity thereafter. These changes are indicative of positional change of aromatic acids of HSA upon its glycation. The tryptophan fluorescence of HSA showed the same pattern as observed in UV spectra presumably involving same aromatic amino acids are involved in both spectral analysis. Glycation causes unfolding of protein leading to exposure of aromatic amino acids towards solvent system resulting in hyperchromicity and gain in tryptophan fluorescence. However, on further incubation up to 28 days causes shielding of aromatic amino acids contributing to hypochromicity and loss of fluorescence intensity. The significant observation in the tryptophan fluorescence was a blue shift on glycation. The results reiterate the earlier observation and suggest conformational changes in glycated HSA. The fluorescence intensity of AGEs was also found to be increased in glycated HSA samples compared to native HSA.

The Far-UV CD spectrum of glycated HSA showed a loss of helical structure as shown by a decrease in the negative ellipticity at 208 and 222 nm. The interaction between native and glycated HSA was confirmed by FTIR spectral analysis as well. Glycation of protein has been shown to result in the protein degradation and/or cross-linking and the observed alterations in the electrophoretic behavior of HSA incubated with glucose apparently results from such effects. Glycated HSA migrated as highly diffuse band with the increase in incubation time and showed the presence of aggregates. However, native HSA migrated as a single band of 66kD molecular weight. Next, colorimetric estimations were carried out to support the biophysical analysis. Ketoamine level at 7 days of incubation was found to be significantly higher in glycated HSA as compared to native HSA, which showed negligible ketoamine content. Levels of carbonyl groups were also

elevated in glycated HSA, an important marker of both glycation and oxidative stress. Number of free amino groups in glycated HSA samples was found to be decreased as compared to native HSA. On the basis of above observations we can infer that incubations with glucose for up to 28 days resulted in a time dependent modification of HSA. Thus, prolonged exposure of HSA to glucose exerts greater deleterious effects on its structure and formation of aggregates.

Hyperglycaemia and accumulation of AGEs due to non-enzymatic glycation of proteins in tissues and serum have important roles in diabetic complications. Moreover, Amadori product is the principal form of glycation mediated modification in proteins. Recent investigations have shown that elevated concentrations of Amadori products play a substantial role in diabetes-related complications. Although there have been important advances in the control of the hyperglycaemia of diabetes by means of diet, hypoglycaemic drugs, insulin and islet transplantation. The long term complications of diabetes such as cataract, nephropathy, retinopathy and atherosclerosis are still leading causes of death. These complications are a direct result of protein alteration which results in irreversible tissue damage. Thus, inhibition of the formation of AGE is believed to play a role in the prevention of diabetes-related complications. Designing a drug having anti-AGE activity is a challenge due to the complexity of reaction involved in the formation of AGE. The most studied and successful agent has been a nucleophilic hydrazine compound viz. aminoguanidine (AG) has shown promising results in-vitro and in animal models in terms of inhibition of AGE formation. A number of other agents such as pyridoxamine, carnosine, taurine and phenyl thiazolium bromide have also been investigated in several studies and have shown promising results. However, except pyridoxamine, none has progressed as yet to the stage of clinical trials. Although some recent studies highlighted the antiglycating potential of a few natural sources, namely garlic, green tea and tomato adequate work has not yet been done. Thus there is a need for developing new antiglycating agents combining higher levels of efficacy, selectivity and safety in humans. Therefore, the identification of antiglycation compounds is attracting considerable interest. Many dietary agents, particularly spices, are a major part of traditional medicine that has been practiced to control many chronic ailments including diabetes.

TQ, TL and EU are commonly used commodities of diet and/or traditional medicine. Therefore, inhibitory effect of varying concentrations of TQ, TL and EU on glycation to HSA induced by glucose was evaluated up to 28 days in-vitro. The interaction of HSA with glucose in presence of these phytochemicals was studied by absorbance, fluorescence and FTIR techniques. Both hyperchromicity and hypochromicity was found to be decreased in presence of aminoguanidine (AG) and at all concentrations of TQ, TL and EU. Eugenol (0.6 μM) showed highest reduction in hyper and hypochromicity in comparison to other formulations of its group. The reduction in conformational changes

due to glycation in presence of AG and phytochemicals were evident at all time points. It indicates that the TQ, TL and EU inhibit the glycation of HSA as observed by a reduction in the hyperchromicity at 7 and 14 days and decrease in hypochromicity at 21 and 28 days. Fluorescence spectra of HSA also exhibited the same pattern as observed in UV spectra. The characterization study of AGEs was performed using AGE-specific fluorescence and quantitation by free lysine side chains. Our results indicated that these phytochemicals at all concentrations inhibit the glycation of HSA as observed by a reduction in the formation of fluorescent AGEs at 14, 21 and 28 days of incubation. Further, formation of new peaks on addition of AG, TQ, TL or EU in the infra-red region confirms the interaction of these phytochemicals with HSA as observed by FTIR spectra.

Ketoamines are early non-enzymatic glycation adducts and are important precursors of AGEs and hydroxyl radicals. Inhibition of ketoamine formation in glycated HSA in presence of AG or varying concentrations of TQ, TL and EU was observed significantly at 7 days. Middle concentration of EU tested i.e. 0.6 μM showed maximum inhibition of ketoamine formation hence, helpful in reducing the formation of glycation induced intermediary compounds. Ketoamines were converted to protein bound carbonyl groups via a protein enediol reaction. The generation of carbonyl groups serves as a marker of protein glycoxidation. The presence of AG and these phytochemicals in glycated HSA significantly reduce the level of carbonyl content at 7, 14, 21 and 28 days. Further these phytochemicals reduced the amount of modified lysine side chains as compared to the control. Free radical generation during glycation was confirmed by quantitation of superoxide radicals in presence and absence of AG, TQ, TL, EU and SOD. The results indicate that early glycation generates free radicals which were quenched significantly by these phytochemicals and AG. Studies with these phytochemicals showed inhibition of different parameters in glycated HSA samples showing a definite role of ROS in the modification of glycated HSA and AGE formation.

Recent evidences suggest that increased oxidative damage as well as reduction in antioxidant capacity could be related to the complications in patients with type-2 diabetes. Thus, the study was extended further to evaluate the antioxidative and antiglycative role of TQ (30 μM), TL (30 μM) and EU (0.6 μM) in diabetic patients with secondary complications. We observed the changes in MDA, protein carbonyl, FRAP, glutathione (GSH) levels, protein crosslinking and/or fragmentation in sera of these patients incubated in presence and absence of these phytochemicals for 21 days. Sera of healthy individual without any treatment served as control. Malondialdehyde (MDA) and protein carbonyl levels were evaluated to determine the lipid and protein damage in serum. FRAP value and glutathione level was taken as the indicator of total antioxidation potential. MDA and carbonyl content was found significantly increased which strongly supported the increased oxidative damage in case of diabetic patients as compared to healthy subjects. In-vitro treatment with these phytochemicals showed a significant

decrease in their level in type-2 diabetic patients. The level of reduced glutathione was significantly lower in the type-2 diabetic patients as compared to normal subjects. On the in-vitro treatment with phytochemicals there was increase in its level in type-2 diabetic patients. FRAP value in serum was also lowered significantly in type-2 diabetic patients. Phytochemical treatment ameliorates FRAP value. Decrease in MDA and carbonyl content with a concomitant increase in GSH and FRAP levels on in-vitro treatment with phytochemicals were found to be more pronounced in the case of eugenol at 0.6 μM followed by thymol (30 μM) and thymoquinone (30 μM).

Serum profile of diabetic patient shows extensive HSA cross-linking, fragmentation and aggregate formation as observed by diffusion of band but the sera incubated with TQ, TL and EU exhibited inhibition of HSA cross-linking, fragmentation and aggregate formation. Amongst the three phytochemicals, EU at the 0.6 μM concentration could inhibit diffusion of band better than 30 μM of TQ and TL. The intake of these phytochemicals may be helpful in diabetes related complications.

All the three phytochemicals described in the present study have cumulative effect of antioxidant and antiglycation activities that might contribute to effective action. However, the in-vitro results may not reflect the effects of these agents in-vivo as they undergo biotransformation process followed by the liver first pass effect, which invariably affect the content, activity and bioavailability of these compounds. Hence, further investigations are needed to address these issues. Various substances included in the present study are naturally occurring. This fact and the results of the present study indicate the possibility of therapeutic use of these phytochemicals for the prevention of diabetic complications. An important therapeutic factor worth of consideration is to administer the phytochemical to diabetic patients. This is essential because it has been proposed that once the progress of excessive glycation has begun, subsequent remediation of hyperglycaemia would not prevent diabetes related complications.

Introduction

Glycation is a non-enzymatic reaction of the amino groups of amino acids, peptides, and proteins with carbonyl group of reducing sugars resulting in the formation of complex brown pigments and protein-protein crosslinks. It is occurring slowly but continuously in cells of all living organisms. It was first studied under defined conditions by Louis Camille Maillard in the early 1900s (Maillard and Gautier, 1912). Thus it came to be known as the Maillard reaction (Fig. 1). Exact realization of the importance of Maillard-like reactions *in-vivo* began in the mid-1970s when studies was done on haemoglobin A_{1c} (HbA_{1c}), a naturally occurring minor human haemoglobin that is elevated in diabetics (Bookchin and Gallop, 1968). HbA_{1c} was known to be a post-translational adduct of glucose with the N-terminal valine amino group of the β chain of hemoglobin, in which the glucose was thought to be attached via non-enzymatically formed Schiff base structure. We found that measurement of the elevation of HbA_{1c} in diabetics allowed assessment of the degree of glucose control integrated over several weeks (Koenig *et al.*, 1976). Later on, the significance of the complex, late stage Maillard processes was recognized as mediators of several complications in diabetes (Bunn *et al.*, 1978) and aging (Monnier and Cerami, 1981). The Maillard reaction is actually a complex series of reactions and is sub-divided into three main stages: early, intermediate, and late.

Early Stage:

The nucleophilic addition reaction between a carbonyl group from a reducing sugar (e.g. glucose, fructose, galactose, mannose etc) and a free amino group is initiated with the reversible formation of an adduct known as Schiff base by conversion of the aldehydic carbon–oxygen double bond of the sugar to a carbon–nitrogen double bond with the amine. This reaction occurs over a period of hours. The Schiff base is a thermodynamically unstable form in relation to the equilibrium cycled pyranose or furanose forms. Therefore, the Schiff base gives rise to an enaminol intermediate by rearrangement and, subsequently, to a relatively stable ketoamine compound/Amadori compound and heyns product in case of fructose. Since this reaction does not require the participation of enzymes, the variables which regulate it *in vivo* are the degree and duration of hyperglycemia, the half-life of the protein, its reactivity in terms of free amino groups, and cellular permeability to glucose. In *in vivo* conditions, the Amadori

product reaches equilibrium after approximately 15–20 days and, through irreversible links, accumulates on both short-lived and long-lived proteins (Lapolla *et al.,* 2005).

Intermediate Stage:

In the second stage, the Amadori compound further undergoes a series of dehydration and fragmentation reactions generating a variety of carbonyl compounds with some formation of initial cross-linked protein species. Carbonyl compounds are generally more reactive than the original carbohydrate and act as propagators by reactions with free amino groups. Among the most active enhancers of the reaction are α-dicarbonyls such as methylglyoxal, glyoxal, glucosones, deoxyglucosones and dehydroascorbate (Thornalley *et al.,* 1999).

Late Stage:

In the late stage, these propagators again react with free amino groups and, through oxidation, dehydration and cyclization reactions, form yellow-brown, often fluorescent, insoluble, irreversible compounds, usually called Advanced Glycation End-Products (AGEs), sometimes known as "melanoidins", which accumulate on long-lived proteins and cause damage and extensive protein cross-linking. AGEs are characterized by a wide structural and physicochemical diversity.

Reducing sugars other than glucose can participate in glycation and do so much faster than glucose, which is the least reactive of all sugars. This may explain why glucose has been selected as the major metabolic sugar during evolution (Bunn and Higgins, 1981). Glucose is the major metabolic sugar present in our body. Even in euglycemic normal individual the level of glucose in plasma is between 65 mg/dl to 100 mg/dl. Therefore even in non-diabetic euglycemic individuals' plasma and other proteins are regularly interacting with glucose and result in formation of glycated end products such as AGEs. Wolffenbuttel *et al.,* (1996) suggested that modification of hemoglobin by advanced glycosylation end products would be a better index for long term glycemia in diabetic patients. While glycation can be detected in physiologic conditions like aging, the reactions are considerably faster and more intensive in the pathophysiologic conditions like the uncontrolled diabetes mellitus associated with persistently elevated blood glucose concentration (Thornalley, 2003). In addition to the multiple pathologies mediated by the *in-vivo* generated AGEs, various exogenous sources such as diet and smoking may also

add significantly to the damage caused by those generated in the body (Koschinsky *et al.*, 1997).

Recent data showed that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites, i.e. alpha-oxoaldehydes, also creatively participate in non-enzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyolic pathway and can also be formed by autoxidation of carbohydrates (glyoxal). As compared to glycation reactions involving molecules like nucleic acid and lipids, protein glycation has been studied extensively showing numerous structural alterations including exposure of thiols, protein compaction, cross linking, fragmentation and susceptibility to proteolysis (Seidler and Seibel, 2000). Glycation by various sugars of a limited number of amino groups in proteins like hemoglobin, albumin and low density lipoproteins induce number of alterations in proteins and loss of biological activity (Turk, 2001). These include conformational alterations, exposure of hydrophobic residues and thiols, loss in allosteric sensitivity (Bunn and Briehl, 1970), ligand binding (McDonald *et al.*, 1979) and receptor recognition. In diabetes mellitus, protein glycation and glucose auto-oxidation may generate free radicals, which in turn catalyzes lipid peroxidation (Baynes, 1991).

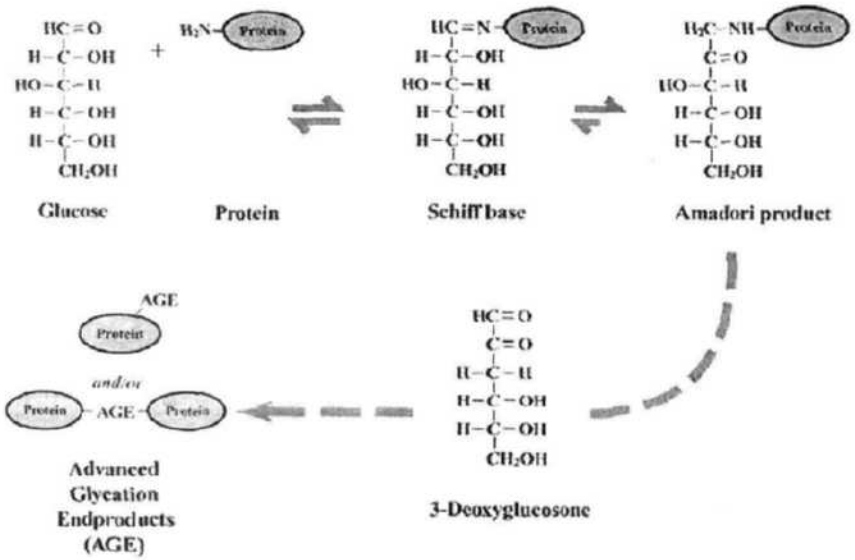

Fig. 1: Glycation of a protein by glucose and subsequent formation of AGEs (Ahmed, 2005).

ADVANCED GLYCATION END PRODUCTS (AGEs):

AGEs are complex, heterogenous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. The formation of AGEs *in-vitro* and *in-vivo* is non-enzymatic and dependent on the turnover rate of the chemically modified target, time and sugar concentration. Persistent hyperglycemia induces abnormal changes such as increase of advanced glycation end products (AGEs) formation, increase of polyol pathway flux, and activation of protein kinase C isoforms (Brownlee, 2001; Evans *et al.*, 2002). Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds. These structures then accumulate during the lifespan of the protein on which they have been formed. For example, lens crystalline proteins (Stevens *et al.*, 1978), insulin (Dolhofer and Wieland, 1979), proteins of erythrocyte membrane (Miller *et al.*, 1980), bovine serum albumin (Arakawa and Timasheff, 1982), human serum albumin (Shaklai *et al.*, 1984), enzymes (Coradello *et al.*, 1982), high and low density lipoproteins (Kirstein *et al.*, 1990), peripheral nerve myelin (Greene, 1983), elastin (Baydanoff *et al.*, 1987) and immunoglobulin G (Newkirk *et al.*, 2003).

CLASSIFICATION OF AGES:

AGEs are usually grouped into fluorescent, non-fluorescent compounds and cross linking AGEs. Some authors also grouped them into toxic and non-toxic AGEs. Toxic AGEs seem to derive from glycolaldehyde or glyceraldehyde and their structures remain to be elucidated (Sato *et al.*, 2006). Many AGEs fluoresce under UV light and are capable of intra and inter-molecular cross-linking, but not all share these properties (Wautier and Schmidt, 2004). On the basis of these two properties AGEs can be classified into three categories (Fig. 2).

1. ***Fluorescent AGE crosslinks:***– Protein-protein crosslinks by these structures *in-vivo* are thought to be responsible for a major share of the deleterious effects of AGEs in diabetes and aging. Along with brown colour, fluorescence is one of the qualitative properties classically used to estimate these AGEs. For example, pentosidine, crossline, pentodilysine, vesperlysine A, B and C. Pentosidine was first isolated and identified FRom dura mater collagen and has since been identified in many tissues (Sell *et al.*, 1991).

2. ***Non-fluorescent AGE crosslinks:-*** Although their ease of detection makes them useful markers of AGE formation, the fluorescent AGE cross-links are thought to account for only one percent or less of the total cross-linking structures formed under physiological conditions (Dyer *et al.*, 1991). Thus, the major AGEs responsible for protein-protein cross-linking *in-vivo* are non-fluorescent structures that have not yet been conclusively identified. The structure of three common examples of this class are imidazolium dilysines, alkyl formyl glycosyl pyrrole and arginine-lysine imidazole.
3. ***Non-crosslinking AGEs:-*** Besides the cross-linking AGEs, a number of non-crosslinking AGEs have been reported under physiological conditions. They may have deleterious effects as precursors of cross-links or as biological receptor ligands causing a variety of adverse cellular and tissue changes. Pyrraline, carboxymethyllysine and imidazolones are examples of non-crosslinking AGEs.

AUTOXIDATIVE GLYCATION AND GLYCOXIDATION:

Oxidation processes are important in the formation of many AGEs (Lapolla *et al.*, 2005). Two routes have been proposed for AGEs formation. The first involves auto-oxidation of free sugar. Monosaccharides, like glucose, exist in equilibrium with their enediol, which can undergo autoxidation in the presence of transition metals to form an enediol radical (Wolff and Dean, 1987). This radical reduces molecular oxygen to generate the superoxide radical ($O_2^{\bullet-}$) and becomes oxidised itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoimine. This is referred to as autoxidative glycation and is outlined in Fig. 3(a). Ketoimines are similar to, although more reactive, than Amadori products and participate in AGE formation.

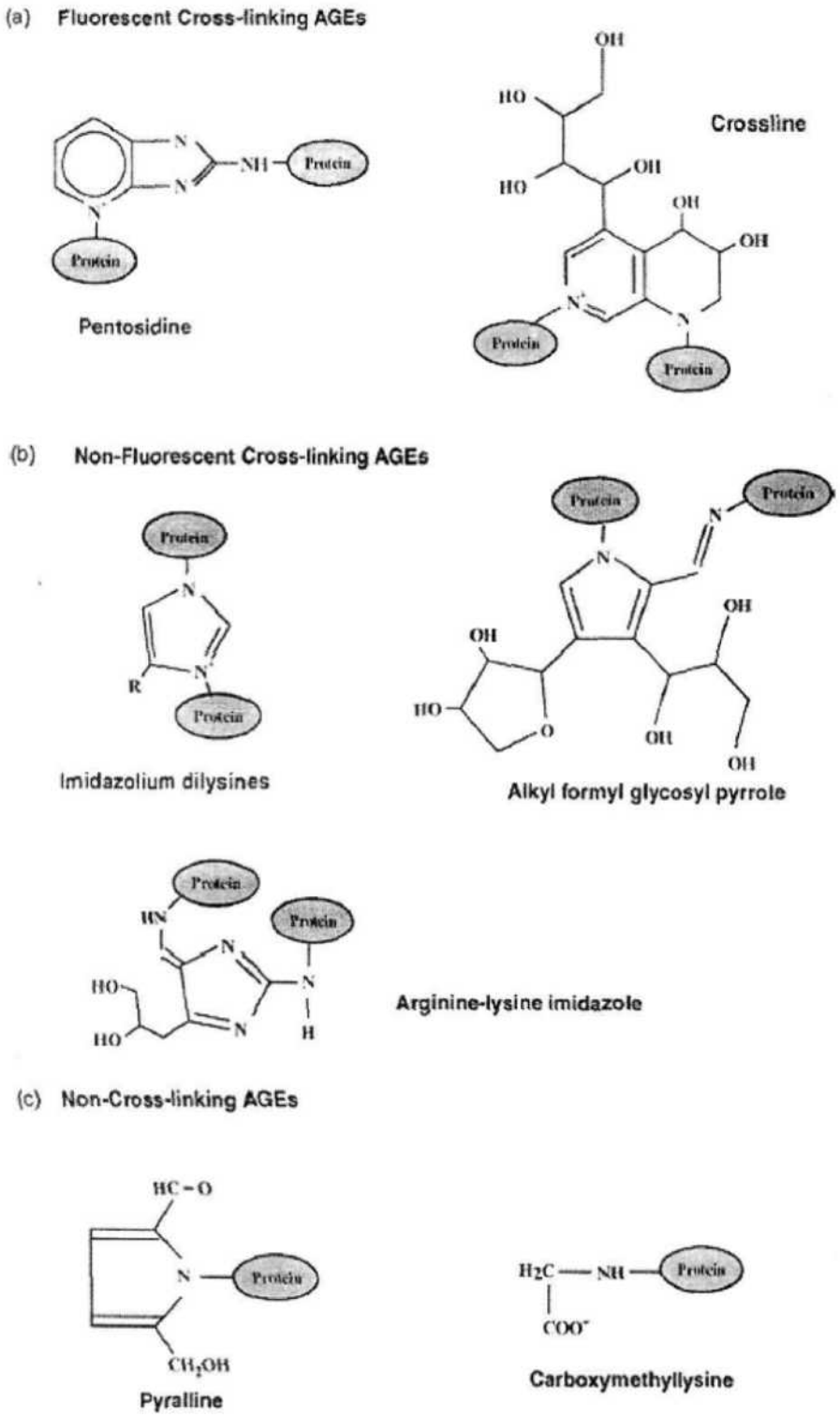

Fig. 2: Chemical structure of (a) Fluorescent cross-linking AGEs, (b) Non-fluorescent cross-linking AGEs, (c) Non-cross-linking AGEs (Ahmed, 2005).

The second mechanism involves autoxidation of Amadori products to AGEs as shown in Fig. 3(b). Protein-bound products of the Amadori pattern, in the presence of molecular oxygen and transition metals, are oxidized and give origin to highly reactive protein-enediols generating protein-dicarbonyls and superoxide radical. The protein dicarbonyl compounds can participate in AGE formation and referred to as glycoxidation products. Once formed, the superoxide radicals can be converted to highly reactive hydroxyl radical via the fenton reaction. ROS such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ may contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process also includes oxidative steps and is therefore called glycoxidation (Bousova *et al.*, 2005; Elgawish *et al.*, 1996; Yim *et al.*, 1995)

RECEPTORS FOR AGES (RAGE)

A number of AGE receptors (RAGE) have been identified in macrophages, endothelial and several other types of cells (Skolnik *et al.*, 1991). Phagocytic cells expressing RAGE internalize and digest AGE modified proteins and therefore these receptors are implicated in protein turnover, tissue remodeling and inflammation (Schmidt *et al.*, 2001; Vlassara, 2001). Expression of RAGE is enhanced in certain cells during diabetes and inflammation. Interaction of AGE with their cellular receptors generates intracellular oxidative stress resulting in the activation of the transcription factor NF-kB and subsequent gene expression which is relevant in diabetic complications (Zill *et al.*, 2001). NF-kB modulates gene transcription for endothelin-1, tissue factor and thrombomodulin and generation of pro-inflammatory cytokines such as interleukin-1a, interleukin-6 and tumor necrosis factor-α (Neumann *et al.*, 1999). There is also enhanced expression of adhesion molecules including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, in addition to other effects such as increased vascular permeability. The intracellular signaling pathways following activation of RAGE by AGEs are outlined in Fig. 4. The importance of oxidative stress, following binding of AGEs with RAGE in endothelial cells was demonstrated by a study which showed that NF-kB and haem-oxygenase messenger RNA, both markers of oxidative stress, become activated. The same study also showed increased oxidative stress in animals after infusion of AGEs (Yan *et al.*, 1994).

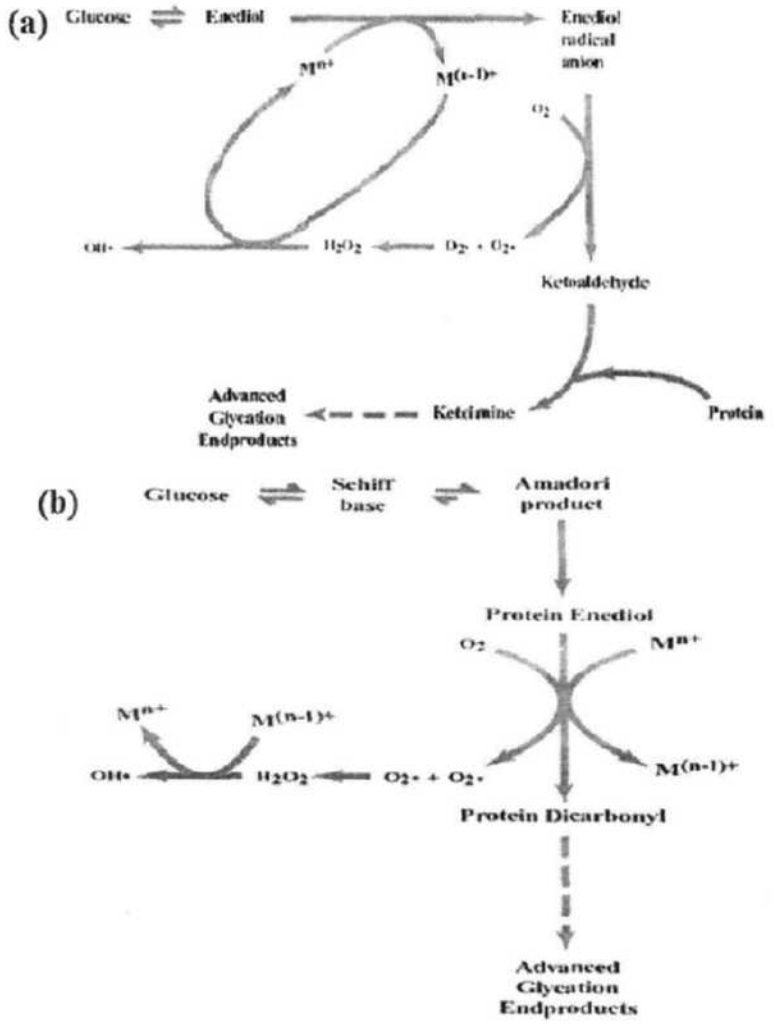

Fig. 3: Reaction schemes for glucose autoxidation (a) and glycoxidation (b) (Ahmed, 2005).

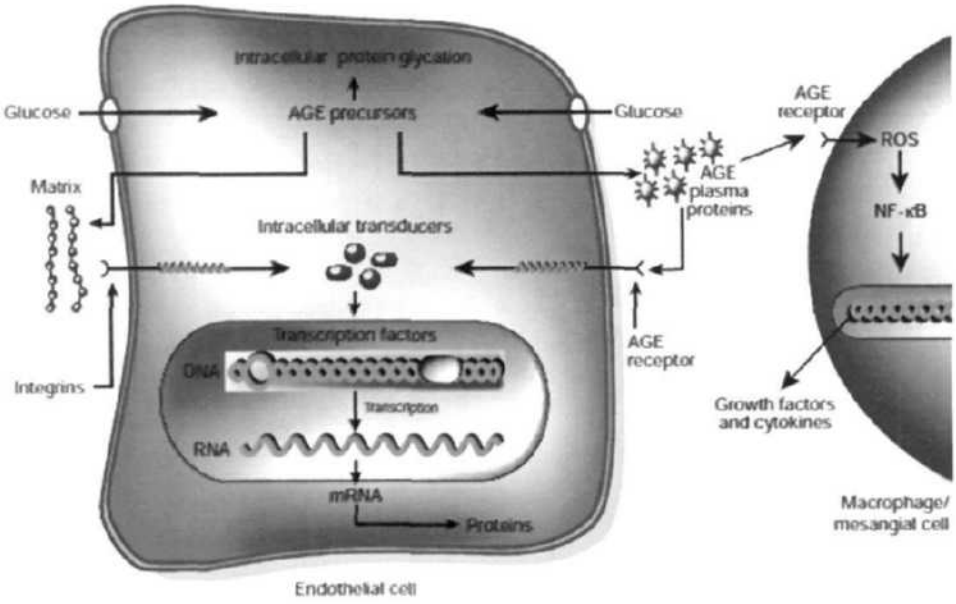

Fig. 4: Mechanism of action of AGEs formed intracellularly (Brownlee, 2001).

BIOLOGICAL EFFECTS OF AGE FORMATION:

AGEs are formed in excess during aging, diabetes mellitus and renal failure (Schleicher *et al.*, 1997). AGE modifications influence the structural as well as functional properties of proteins. Due to AGE modifications, several enzymes alter their activity. Methylglyoxal-modified serum albumin exhibits impaired esterase activity compared to unmodified albumin (Ahmed and Thornalley, 2005). Moreover cystein proteases like cathepsins are inhibited by methylglyoxal modification at active site of cysteins (Zeng and Davies, 2005). Glycated and crosslinked proteins exhibit an increased resistance to proteasomal protein degradation, and hence results in markedly enhanced biological half-life (Bulteau *et al.*, 2001). In contrast to intracellular proteins, collagen in the extracellular matrix (ECM) has a relatively long biological half-life and is directly exposed to high levels of glucose outside the cell. Indeed, modified collagen becomes more resistant to degradation by metalloproteinases which cause accumulation of AGE-modified collagens in the ECM which mediate its stiffening leading to heart and vessel dysfunction (Badenhorst *et al.*, 2003). Lens crystallins are also a long-lived target, leading to cataracts. Damage to DNA due to AGE formation may cause birth defects (Ulrich and Cerami, 2001). Thus AGEs appear to damage cells by three mechanisms (Fig. 5):

The first is the modification of intracellular proteins/intracellular glycation including, most importantly, proteins involved in the regulation of gene transcription (Giardino *et al.*, 1994; Shinohara *et al.*, 1998). The second mechanism being, these AGE precursors can diffuse out of the cell and modify adjacent extracellular matrix molecules nearby/cross-link formation (McLellan *et al.*, 1994) with changes signaling between the matrix and the cell causes cellular dysfunction (Charonis *et al.*, 1990). The third mechanism being, these AGE precursors diffuse out of the cell and modify circulating proteins in the blood, such as albumin. These modified circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory :ytokines and growth factors, which in turn cause vascular pathology (Smedsrod *et al.*, 7; Vlassara *et al.*, 1995).

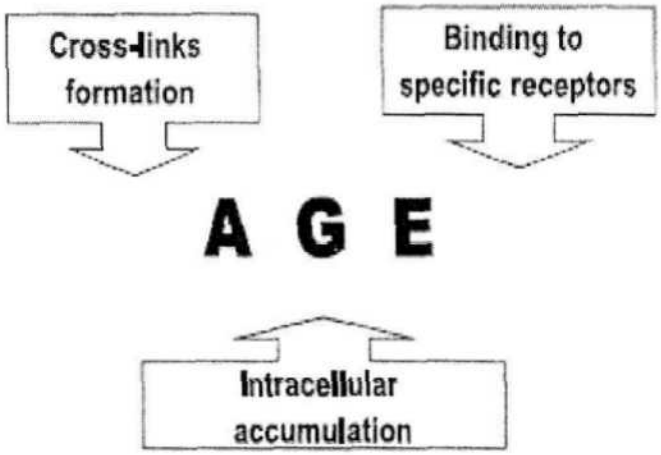

Fig. 5: Harmful effect of AGEs (Lapolla *et al.*, 2005).

The major ways in which glycation changes protein functions are by:

a) Inhibition of regulatory molecule binding
b) formation of cross linkage of glycated protein
c) trapping of glycated proteins by extracellular matrix
d) decreasing the susceptibility of protein to proteolysis
e) loss of biological activity of enzymes including malate dehydrogenase, glucose-6-phospahte dehydrogenase, glutathione reductase, glyceraldehyde-3-phospahte dehydrogenase, catalase and superoxide dismutase (Heath *et al.*, 1996).
f) abnormality of nucleic acid function
g) increased immunogenicity in relation to immune complex formation (Turk, 2001).

GLYCATION OF HUMAN SERUM ALBUMIN:

Human serum albumin is a major protein component of the serum. Albumin contains 585 amino acids and has a molecular weight of 66 kD. This globular protein contains 18 tyrosines, six methionines, one tryptophan, 17 disulphide bridges, and only one Free cysteine, (Cysteine-34) (Sugio *et al.*, 1999). Structurally it consists of 67% of the secondary structure which comprises 28 α-helical segments. Rest of the secondary structure consists of 10% β-turns and 23% extended peptide chain. The tertiary structure of HSA is arranged in heart shape in three homologous domains I, II, III (Fig. 6). It

contains only one tryptophan located in domain II (Coussons *et al.*, 1997; Weber, 1975). This highly soluble protein is present in human plasma at normal concentrations between 35 and 50 g/L. Human serum albumin is a member of the multi-gene family of proteins that include α-fetoprotein and human-group specific component. In normal conditions, its half-life is about 20 days, and its plasma concentration represents equilibrium not only between its synthesis in the liver and its catabolism, but also its transcapillary escape. It is produced in the liver at a rate of nearly 0.7 mg/g of liver tissue per hour (Peters, 1970). Its production is under the control of insulin and somatotropin (Hutson *et al.*, 1987). Albumin has several important physiological and pharmacological functions. It transports metals, fatty acids, cholesterol, bile pigments, and drugs. It is a key element in the regulation of osmotic pressure and distribution of fluid between different compartments. Its most striking property is its ability to bind an unusually broad spectrum of ligands (Brown, 1982). These include inorganic cations, organic anions, various drugs, amino acids, and perhaps most important, physiologically available hydrophobic molecules like bilirubin, hemin, and fatty acids. As a result, albumin is considered a multifunctional plasma transport protein.

Albumin is also responsible for storage and transport of a large number of drugs in the plasma (Bhattacharya *et al.*, 2000). It is also supposed to have a high affinity to metal ions such as Cu^{2+} and Zn^{2+} and act as an antioxidant in the vascular compartment due to its scavenging of reactive oxygen and nitrogen species generated due to basal aerobic metabolism normally and formed at an increased rate during inflammation (Halliwell and Gutteridge, 1990). It is present in the serum in a soluble form and is a major contribute to 80% of plasma colloid pressure (Lundsgaard-Hansen, 1986).

It also acts as an important Acid-Base buffer in plasma. HSA non-enzymatically reacts with glucose to form a stable glycated albumin (Shaklai *et al.*, 1984). This process is especially elevated in Diabetes (Dolhofer and Wieland, 1980) due to increased glucose concentration in plasma. This process of non-enzymatic glycation proceeds in a glucose concentration, incubation period and temperature dependent manner (Baynes *et al.*, 1984). The principle site of glycation of HSA is lysine-525, but the lysine residues in positions 199, 281, and 439 are also susceptible to glycation. In addition there are six more residues that glycate but with much less efficiency (Shaklai *et al.*, 1984).

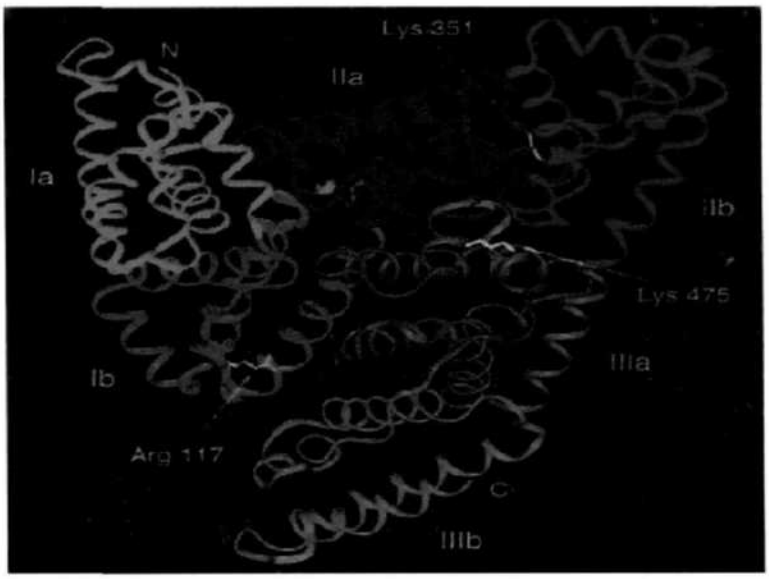

Fig. 6: Schematic drawing of the HSA molecule. Each subdomain is marked with a different colour (yellow,Ia; green, Ib; red, IIa; magenta, IIb; blue, IIIa; and cyan IIIb). N- and C- termini are marked N and C, respectively. Arginine 117, lysine 351 and lysine 475 which may be sites for binding long-chain fatty acids are colored white (Sugio *et al.*, 1999).

The *in-vitro* exposure of protein to glucose results in the non- enzymatic covalent attachment of glucose to lysine side chains in a manner that observed *in-vivo*. HSA is typically three times more glycated than the rest of the population in conditions of hyperglycemia (Bourdon *et al.*, 1999). In diabetes, HSA may rise from 6-10% to 20-30% and hence serves as the indicator of glycation (Nakajou *et al.*, 2003). HSA also represents the major and predominant circulating antioxidant in plasma known to be exposed to continuous oxidative stress (Soriani *et al.*, 1994). Glucose and free radicals were found to impair the antioxidant properties of the serum albumin (Bourdon *et al.*, 1999). Many studies show the presence of elevated levels of oxidized albumin, in patients with diabetes mellitus (Suzuki *et al.*, 1992), aging (Era *et al.*, 1995), patients with chronic hepatitis C (Rigamonti *et al.*, 2003), oxidized albumin is a reliable marker of oxidative stress in hemodialysis patients (Mera *et al.*, 2005) and many other diseases.

INHIBITION OF GLYCATION:

Body has several humoral and cellular defence mechanisms to protect tissues from deleterious effects of glycation reaction and AGE accumulation. These include the glyoxalase systems (I and II) that catalyses the deglycation of methylglyoxal to D-lactate (Thornalley, 1998). The discovery of deglycating enzymes has implications for the repair of protein damage by fructose (Monnier, 2005). A variety of plasma amines may react with sugar and Amadori carbonyl groups to reduce AGEs. Numerous compounds have been investigated for anti-glycation activity and the various sites where potential anti-glycation or AGE compounds could act are outlined in Fig. 7. Currently several strategies are employed to control protein glycation.

(i) Block free amino groups on proteins, preventing glycation by free sugars. However, the biological effect of reducing free protein amino groups is not known.

(ii) Block carbonyl groups on reducing sugars, Amadori products and dicarbonyl intermediates (3-deoxyglucosone, methylglyoxal, etc.) effectively reducing glycation and/or AGE formation. Again, the effect of reducing available carbonyl groups *in-vivo* may not be desirable.

(iii) Antibodies may be used to block Amadori products. This approach has the advantage of specificity compared to use of compounds that merely recognize carbonyl groups.

(iv) Chelation of transition metals by ceruloplasmin may reduce glycation-derived free radicals. However, many transition metals have important physiological functions and their complete removal may have undesirable consequences.

(v) Antioxidants may protect against free radicals derived via autoxidative glycation, glycoxidation and AGEs.

(vi) Enzymes (Amadoriases) may be used to deglycate Amadori products or inactivate intermediates such as 3-deoxyglucosone.

(vii) AGE-cross-link breakers offer the potential of reversing diabetic complications although their precise mechanism of action is still unclear.

(viii) RAGE blockers could prevent interaction of AGEs with RAGE to suppress the cellular and inflammatory changes associated with the development of diabetic complications.

DIABETES:

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with disturbances of fat, carbohydrate and protein metabolism resulting from defects in insulin secretion and/or insulin action (Turko *et al.*, 2001). The recent statistics indicate that the global prevalence of DM, estimated as 366 million in 2011, will increase up to 522 million by 2030 (Whiting *et al.*, 2011). Diabetes is the fifth leading cause of death in the US and the number of people with diabetes in the world is expected to approximately double between 2000 and 2030 (Wild *et al.*, 2004). India has world's largest number of diabetic subjects and the prevalence of diabetes and impaired glucose tolerance were 12.1% and 14.0% respectively, with no gender difference (Ramachandran *et al.*, 2001).

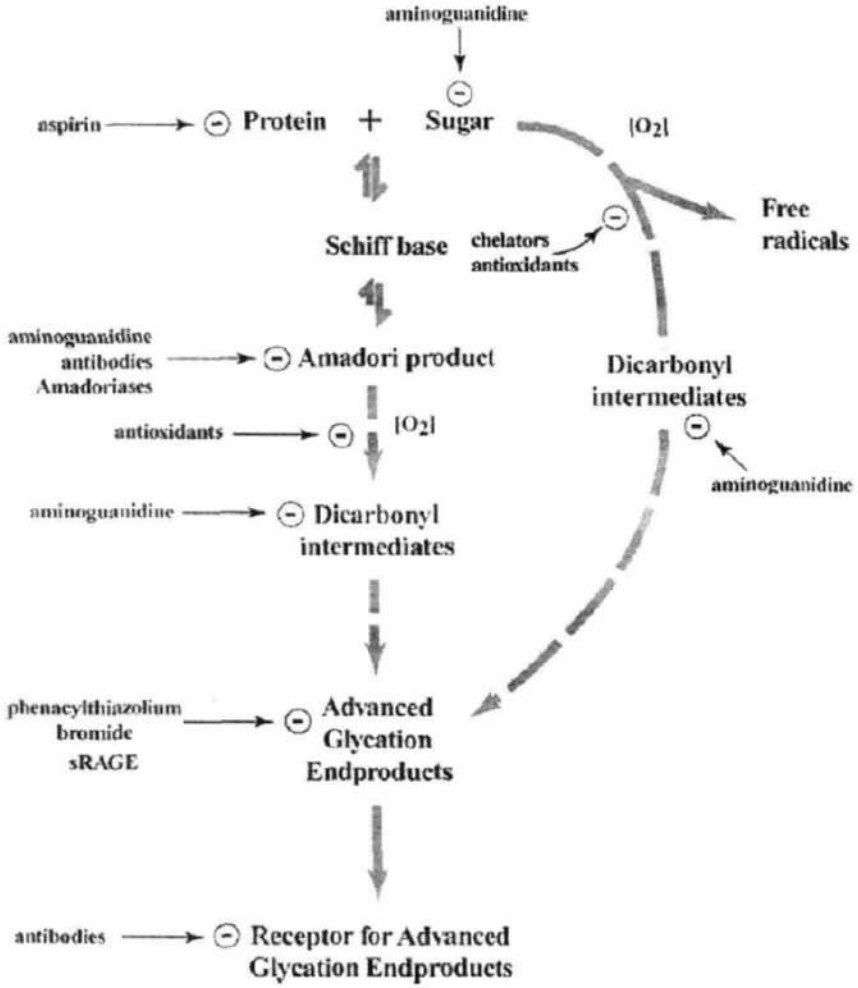

Fig. 7: Potential sites where pharmacological compounds may act to inhibit protein glycation and AGE-mediated damage (Ahmed, 2005).

The prevalence of diabetes in India is about three times higher in urban population compared to rural population and also the prevalence of diabetes varies widely across the nation, a very high prevalence (16.3%) was reported in Thiruvanathapuram in Kerala State in the year 1999, in the same year, a prevalence of 8.3 per cent was reported from Guwahati (Moebus *et al.*, 2010; Ramachandran *et al.*, 2001). Depending on the etiology of DM, factors contributing to hyperglycemia may include reduced insulin secretion, decreased glucose utilization and increased glucose production (Kasper *et al.*, 2005). Distinct types of diabetes mellitus (DM) are caused by complex interactions of genetics, environmental factors and life style choices. In the United States, DM is the leading cause of end-stage renal disease, non-traumatic lower extremity amputations and adult blindness.

CLASSIFICATION OF DIABETES:

Classification of DM is on the basis of pathogenic process that leads to hyperglycemia.

- Type 1 diabetes results from β-cell destruction, usually leading to absolute insulin deficiency.
- Type 2 diabetes results from a progressive insulin secretory defect on the background of insulin resistance, impaired insulin secretion and increased glucose production.
- Other specific types of diabetes due to other causes, e.g. genetic defects in β-cell function, genetic defects in insulin action, diabetes of the exocrine pancreas (such as cystic fibrosis), endocrinopathies (e.g. acromegaly, hyperthyroidism) and drug or chemical induced (such as nicotinic acid, protease inhibitors) or due to infections (e.g. congenital rubella).
- Gestational diabetes mellitus include impaired glucose tolerance during pregnancy.

DIABETIC COMPLICATIONS:

The complications of DM affect many organ systems and are responsible for the majority of morbidity and mortality related to the disease. One of the most prevalent metabolic syndromes world-wide, diabetes mellitus (DM), is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular and connective-tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy, neuropathy and complications

of the macro-vasculature such as atherosclerosis; potentially resulting in heart disease, stroke and peripheral vascular disease (Hudson, 2002).

In diabetic subjects, hyperglycemia is widely recognized as the major cause of diabetic secondary complications due to over generation of ROS (Fig. 8) (Palm *et al.*, 2003).

Several hypotheses relating to hyperglycemia have been proposed. Four main hypotheses as shown in Fig. 9 are: (i) Increased polyol pathway flux, (ii) Increased advanced glycation end product (AGE) formation, (iii) Activation of protein kinase C isoforms, and (iv) Increased hexosamine pathway flux.

1. Diabetic retinopathy:

Diabetic retinopathy is one of the most important microvascular complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (L'Esperance et al., 1990). In a large population-based study, prevalence of any degree or proliferative retinopathy was highest in the younger-onset, insulin-taking diabetic patients and lowest in older-onset group not taking insulin (Klein *et al.*, 1984). The prevalence of diabetic retinopathy increases with duration of diabetes.

Diabetic retinopathy involves both morphological and functional changes in the retinal capillaries, including basement membrane thickening, loss of pericytes, increased permeability and vascular dysfunction. AGEs have been detected in retinal blood vessel walls and contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage (Beisswenger *et al.*, 1995). AGEs exert their effect on microvascular endothelial cells and pericytes by upregulating levels of their RAGE messenger RNA (Tanaka *et al.*, 2000). AGEs may cause loss of pericytes and death of endothelial cells in diabetic retinopathy. The role of AGE in the development of diabetic retinopathy and the effect of the AGE-formation inhibitor, aminoguanidine, has been examined in animal models (Hammes *et al.*, 1995).

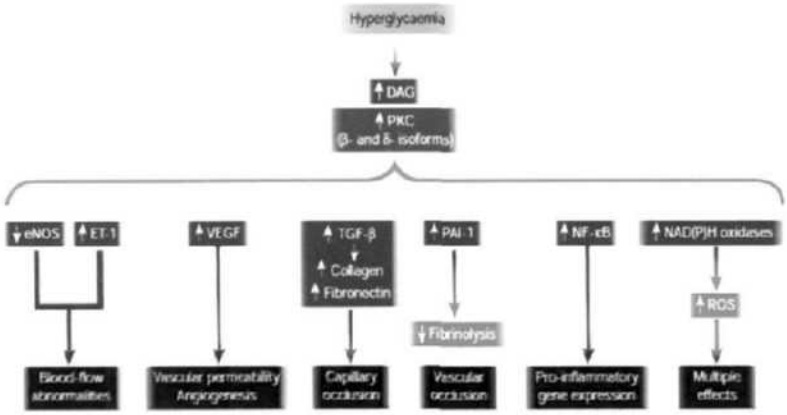

Fig. 8: Diabetic secondary complications due to hyperglycemia (Brownlee, 2001).

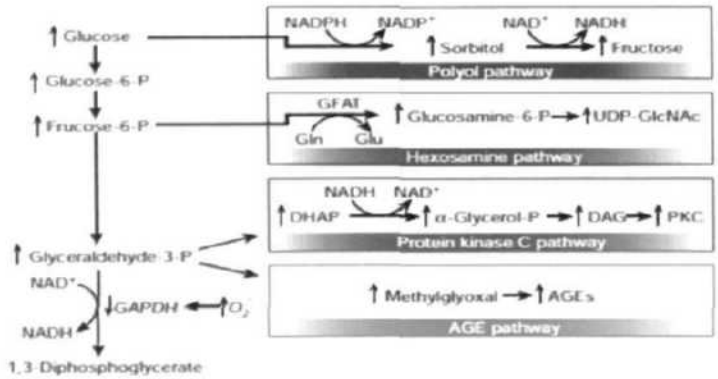

Fig. 9: Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage (Brownlee, 2001).

2. Diabetic nephropathy:

Diabetic nephropathy is a leading cause of end stage renal disease, and accounts for disabilities and the high mortality rate in patients with diabetes (Krolewski *et al.*, 1991). Development of diabetic nephropathy is characterized by glomerular hyperfiltration and thickening of glomerular basement membranes, followed by an expansion of extracellular matrix in mesangial areas and increased urinary albumin excretion rate. Diabetic nephropathy ultimately progresses to glomerular sclerosis associated with renal dysfunction (Sharma and Ziyadeh, 1995).

Serum AGE levels reflect the severity of diabetic nephropathy and their measurement can predict the histopathological conditions (Berg *et al.*, 1997). Circulating serum AGE level is so markedly increased in patients with diabetic nephropathy and renal insufficiency that it cannot be cleared by the kidneys (Turk, 2001). A number of studies have demonstrated that aminoguanidine decreased AGE accumulation and plasmaprotein trapping in the glomerular basement membrane (Raj *et al.*, 2000).

3. Diabetic neuropathy:

Diabetic neuropathies are a family of nerve disorders caused by diabetes. People with diabetes can, over time, develop nerve damage throughout the body. Diabetic neuropathy is associated with risk factors for other vascular complications such as poor metabolic control, dyslipidemia, hypertension, body mass index, smoking, microalbuminuria and retinopathy (Cameron *et al.*, 2001; Forrest *et al.*, 1997; Tesfaye *et al.*, 1996). Both vascular and metabolic factors have been involved in the pathogenesis of diabetic neuropathy. Studies in human and animal models have shown reduced nerve perfusion and endoneurial hypoxia, which might play a role in nerve dysfunction (Ibrahim *et al.*, 1999). An interaction between AGE-myelin and macrophages may initiate or contribute to the segmental demyelination associated with diabetic neuropathy (Vlassara *et al.*, 1984). Aminoguanidine (AG) treatment inhibits an accumulation of fluorescent AGE in diabetic nerves, and partially prevents demyelination and axonal atrophy probably through the correction of endoneurial microcirculation (Sugimoto and Yagihashi, 1997).

4. Diabetic atherosclerosis:

Atherosclerotic arterial disease may be manifested clinically as cardiovascular disease (CVD). CVD is responsible for about 70 % of all causes of death in patients with type-2 diabetes (Laakso, 1999). Conventional risk factors, including hyperlipidemia, hypertension, smoking, obesity, lack of exercise, and a positive family history, contribute similarly to macrovascular complications in type-2 diabetic patients and non-diabetic subjects (Laakso, 1999). AGEs formed on the extracellular matrix results in decreased elasticity of vasculatures, and quench nitric oxide, which could mediate defective endothelium-dependent vasodilatation in diabetes (Bucala *et al.,* 1991). AGE modification of low density lipoprotein (LDL) exhibits impaired plasma clearance and contributes significantly to increase LDL *in-vivo*, thus being involved in atherosclerosis (Bucala *et al.,* 1995). Binding of AGEs to RAGE results in generation of intracellular ROS generation and subsequent activation of the redox-sensitive transcription factor NF-kB which promotes the expression of a variety of atherosclerosis-related genes. Taken together, in diabetes, when fueled by hyperglycemia, AGEs and oxidative stress, the AGE-RAGE axis amplifies vascular stress and accelerates atherosclerosis and neointimal expansion (Naka *et al.,* 2004). Blockade of the AGE-RAGE interaction may lead to a successful reduction of CVD in diabetes. Other study shows a correlation between AGE levels and the degree of atheroma in cholesterol-fed rabbits, and that AG has an anti-atherogenic effect in these rabbits by inhibiting AGEs formation (Panagiotopoulos *et al.,* 1998).

PROTEIN OXIDATION IN DIABETES:

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. These consequences of

oxidative stress can promote the development of complications of diabetes mellitus (Baynes and Thorpe, 1999; Ceriello *et al.*, 2000).

Diabetic patients exhibit elevated levels of intracellular iron and copper ions which in the presence of glycated proteins, have been shown to enhance the generation of free radicals *in-vitro* (Dean *et al.*, 1991). These highly reactive species in turn are able to induce oxidative degradation of protein *in-vitro* (Pacifici and Davies, 1991). Glycation is a major source of ROS i.e., generated by oxidative pathways of glycation (Rahbar and Figarola, 2003). Several studies support the idea that glycation and oxidation are closely linked processes; glucose autoxidation plays an essential role in non-enzymatic glycation of protein. AGEs are important source of free radicals resulting from non-enzymatic glycation and oxidation of proteins and lipids (Thomas *et al.*, 2005). Free radicals and glycation are central to chronic diseases, degeneration and ageing. Overproduction of free radicals accelerates cell ageing and is counteracted by antioxidants. The analysis of mechanism generating free radicals and of the reaction of AGEs with cellular metabolism opens new avenues for the delaying of the development of chronic diseases like diabetes and neurodegenerative diseases (Giardino *et al.*, 1998).

ANTIGLYCATING AGENTS:

The first compound that has been extensively studied *in-vitro* and *in-vivo* to be a powerful inhibitor of AGE formation is aminoguanidine (Brownlee *et al.*, 1986). AG prevents the formation of fluorescent AGEs and glucose derived collagen cross-linking. The mechanism of inhibition of AGE formation by AG involves trapping of reactive dicabonyl intermediates such as methylglyoxal, glyoxal and 3-deoxyglucosone (Thornalley, 2003; Thornalley *et al.*, 2000). In addition to chelating or antioxidant activity, AG also acts as true scavenger of carbonyl compounds (Thornalley *et al.*, 2000). Pyradoxamine, a form of vitamin B6, found to inhibit carboxymethyl lysine formation *in-vitro* but it does not interact directly with Amadori intermediates but interfere with the post amadori oxidative reactions by binding catalytic metal ions (Chetyrkin *et al.*, 2008; Voziyan *et al.*, 2003). Many studies suggested that metal catalyzed oxidation plays a critical role in glucose induced modification in collagen. Transition metals like Cu^{2+} ions can catalyze both glycation and glycoxidation in concentration dependent manner

(Sajithlal *et al.*, 1999). Carnosine appears to possess antiglycating, antioxidant and free radical scavenging activity. Carnosine inhibits inactivation and crosslinking of enzymes including superoxide dismutase glycation (Ukeda *et al.*, 2002) and oxidation (Stvolinskii *et al.*, 2003). It was found recently the imidazolium group of histadine on carnosine stabilizes the adduct formation at the primary amino group and hence it may play an important role for an anti-crosslinking agent (Hobart *et al.*, 2004). Some anti-inflammatory compounds such as acetylsalicylic acid, ibuprofen indomethacin were also reported to inhibit glycation by preventing the oxidative stress associated with the formation of AGE (Caballero *et al.*, 2000; Shastri *et al.*, 1998; Sobal and Menzel, 2000). Aspirin was also found to inhibit pentosidine formation (Fu *et al.*, 1994; Urios *et al.*, 2007). Some anti-diabetic drugs metformin and progiatazone were also reported to be powerful AGE inhibitors (Rahbar *et al.*, 2000). Recently, two new classes of aromatic compounds, derivatives of aryl (and heterocyclic) ureido and aryl (and heterocyclic) carboxamide-phenoxy-isobutyric acids and benzoic acids have been reported to be potent inhibitors of glycation and AGE formation (Rahbar and Figarola, 2003). *In-vitro* studies showed that they could directly interact with several reactive dicarbonyls such as glyoxal and methylglyoxal. They were also found to be potent chelators of Cu^{2+} and therefore can suppress hydroxyl radical production during sugar autoxidation and glycation reactions (Rahbar and Figarola, 2003).

Recent studies have highlighted the possible benefits of using plant extracts for decreasing glycation over the currently used drugs (Rates, 2001). Flavonoids like quercetin and rutin represent the most common and widely distributed group of plant phenolics and are abundant in foods. They show important antioxidant and AGE inhibitory properties according to their structure (Farrar *et al.*, 2007). Quercetin has been shown to attenuate diabetic nephropathy in streptozotocin-diabetic rats (Anjaneyulu and Chopra, 2004). Bonnefont-Rousselot (2004) stated that improved antioxidant status is one mechanism by which dietary antioxidant treatment contributes to the prevention and reduction of diabetic complications (Jung *et al.*,2008).

Garcinol, isolated from Garcinia indica fruit rind has been shown to possess antiglycating property *in-vitro* along with antioxidant and metal chelating properties (Yamaguchi *et al.*, 2000). Mizutani *et al.*, (2000) isolated resveratrol, a natural phytoestrogen found in

grapes which is found to inhibit AGE induced proliferation and collagen synthesis in vascular smooth muscle. Cyperus rotundus suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation (Ardestani and Yazdanparast, 2007). Regarding the significance of glycoxidative stress to diabetic pathology, a supplement of antioxidants to inhibit the process of protein modification appears to be a good strategy for preventing diabetic complications (Rahbar and Figarola, 2003).

FREE RADICAL BIOCHEMISTRY:

Free radicals are the chemical species having unpaired electrons that are generated *in-vitro* as well as *in-vivo*. They are highly reactive entities and remain so until and unless their valence shell electrons get paired and attain stability. They are formed from parent molecules via the breakage of a chemical bond keeping one electron by each of its fragment or by cleavage of a radical to generate another radical. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. Besides, they are also produced in redox reactions (Halliwell and Gutteridge, 2007). Those reactive molecules containing oxygen are termed as reactive oxygen species (ROS). ROS possess modulatory roles on the physiology of body cells and also act as second messengers causing signal transduction (Myatt, 2010; Tavakoli and Asmis, 2012). It encompasses a diverse variety of chemical species including superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), singlet oxygen, hydrogen peroxide (H_2O_2), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$) and hypochlorous acid (HOCl) (Jakus, 2000).

A great deal of literature indicates that free radicals especially active oxygen-centered ones are highly heterogeneous and highly reactive that can invade on the important biomolecules like proteins, lipids and DNA. This consequently can lead to enzyme inactivation, protein modification (deactivation or hyperactivation), lipid peroxidation, membrane degradation, DNA- strand breakage and base modification and so on. Hence, biological free radicals are potentially reactive enough to damage the neighboring biomolecules and can be causative agents for various diseases, aging and cancer (Baek *et al.*, 2003; Hassan *et al.*, 2012; Valko *et al.*, 2004). These radicals are produced by either *endogenous* sources or by *exogenous* sources (Fig. 10). Endogenously, these radicals are

generated from physiological or bodily actions such as immune response, inflammation, toxicity, infection, excessive exercise, ischemia, cancer and aging etc. In analogous to phosphorylation modification of proteins, redox signaling is emerging in reference to events of oxidation of proteins and hence modification by ROS. Indeed, there are many sources of ROS in the cell namely, nicotinamide adenine dinucleotide phosphate oxidase (Block and Gorin, 2012), xanthine oxidase, uncoupling of nitric oxide synthase, cytochrome P_{450} (Cubero and Nieto, 2012; Izyumov *et al.*, 2010). However, one of the main sources of ROS is the mitochondrion within the cell where the $O_2^{\cdot -}$ is produced as a byproduct of normal oxidative phosphorylation. Exogenous sources include pollution (air and water), heavy metals (lead, mercury, cadmium etc.), certain drugs (gentamycin, cyclosporine), smoking and radiations etc. These agents after getting into the body via different routes are decomposed or metabolized and trigger generation of various free radicals (Brodin and Roed, 1984). Three major forms of ROS shall now be individually discussed in short.

Superoxide ($O_2^{\cdot -}$): Superoxide anion is created from molecular oxygen by the addition of an electron. Its production mainly occurs inside the mitochondrion during the electron transport chain, when a small number of electrons escape from electron transport chain complexes I and III (Valko *et al.*, 2007). $O_2^{\cdot -}$ is responsible for the dismutation and release of H_2O_2, which acts as precursors for $^{\cdot}OH$ ion formation by the catalysis of metal atoms (Holley *et al.*, 2010). It lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced. The formation of superoxide takes place spontaneously, especially in the electron-rich aerobic environment with the activity of respiratory chain enzymes like flavoenzymes, lipoxygenase and cycloxygenase (Coughlan *et al.*, 2009). The superoxide radical is produced enzymatically by the reaction with xanthine oxidase.

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric acid} + O_2^{\cdot -} + H^+$$

Hydrogen peroxide (H_2O_2): H_2O_2 is not a free radical but is nonetheless highly important much because of its ability to penetrate biological membranes and is produced by the dismutation of $O_2^{\cdot -}$ or by direct reduction of $O_2^{\cdot -}$ with two electrons (Topo *et al.*, 2010).

$$O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$$

It acts as an intermediate in the production of more reactive ROS molecules including HOCl by the action of myeloperoxidase, an enzyme present in the phagosomes of neutrophils and most importantly, formation of ·OH via oxidation of transition metals. It's important functional role is in intracellular signaling (Sundaresan *et al.*, 1995) and can be removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases and peroxiredoxins (Mates *et al.*, 1999).

$$H^+ + Cl^- + H_2O_2 \longrightarrow HOCl + H_2O$$

Hydroxyl radical (·OH): Due to its strong reactivity with biomolecules , ·OH is probably capable of doing more damage to biological systems than any other ROS (Betteridge, 2000). They are produced by Fenton reaction which involves metal ions like Fe^{2+} and Cu^{2+} with H_2O_2, often bound in complex with different proteins or other molecules (Liu *et al.*, 2012). Transition metals thus play an important role in the formation of ·OH (Halliwell, 1999). Transition metals may be released from proteins such as ferritin and the [4Fe-4S] centre of different dehydrases by reactions with $O_2^{\cdot -}$. This mechanism, specific for living cells, has been called the *in-vivo* Haber-weiss reaction (Fridovich, 1997).

$$H_2O_2 + Cu^+/Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Cu^{2+}/Fe^{3+}$$

- ***Nitric oxide (NO):*** Nitric oxide represents an odd member of the free radical family as it contains unpaired electrons and it is not reactive with various biocellular molecules (Wu *et al.*, 2011). Contrarily it easily reacts with other free radicals (e.g., peroxyl and alkyl radicals), generating mainly less reactive molecules, thus in fact functioning as a free radical scavenger in order to inhibit cellular oxidation of lipids in the cell membranes (Hogg and Kalyanaraman, 1998). The $O_2^{\cdot -}$ and NO react with each other to give $OONO^-$(peroxynitrite), which is highly cytotoxic (Beckman and Koppenol, 1996). $OONO^-$ may react directly with diverse biomolecules in one- or two-electron reactions, readily react with carbon dioxide to form highly reactive nitroso peroxocarboxylate ($ONOOCO_2^-$), or protonated as peroxonitrous acid (ONOOH) undergo homolysis to form ·OH and $^{\cdot}NO_2$ or rearrange to nitrate (NO_3). Peroxynitrite, directly or via its reaction products, may oxidize low density lipoproteins, release copper ions by destroying ceruloplasmin, and generally attack tyrosine residues in different proteins, as observed in many inflammatory diseases (Halliwell, 1997). NO is synthesized enzymatically from L-arginine by NO synthase (Andrew and Mayer, 1999).

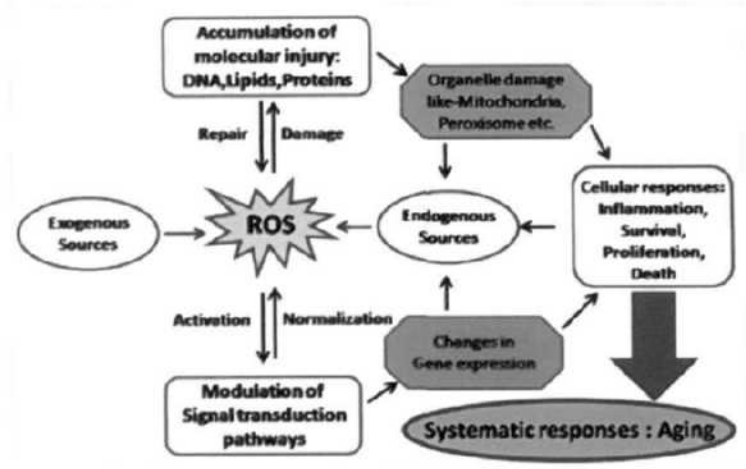

Fig. 10: Reactive oxygen species (ROS) generated by endogenous as well as exogenous sources (Brodin and Roed, 1984).

ANTIOXIDANT DEFENSE SYSTEM:

Free radicals exert derogatory effects on our cellular structure and functions. Hence, living systems have been equipped with antioxidant defense system comprising of endogenous antioxidant molecules or cellular reductants [glutathione (GSH), sulfhydryl groups (-SH), thioredoxin] and antioxidant enzymes:

1. ***Superoxide dismutase (SOD):*** It dismutates $O_2^{\cdot -}$ into H_2O_2 and oxygen. Superoxide dismutases (SODs) are metalloenzymes and their role is to protect aerobic cells against $O_2^{\cdot -}$ action. They catalyze the conversion of superoxide molecules to H_2O_2 and O_2 and therefore form one of the cell's major defense mechanisms against oxidative stress (McCord and Fridovich, 1969).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

2. ***Catalase:*** Catalase protects cells against H_2O_2 generated inside them. It has an important role in the acquisition of tolerance to oxidative and nitrosative stress in cellular adaptive response.

$$2H_2O_2 \rightarrow 2H_2O + \tfrac{1}{2} O_2$$

3. ***Glutathione peroxidase:*** Glutathione peroxidase uses the thiol reducing power of glutathione to reduce oxidized lipids and protein targets of ROS. Glutathione Peroxidase

catalyses hydroperoxide reduction using GSH, thus protecting mammalian cells against oxidative damage.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

4. ***Glutathione reductase:*** Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase. This system traps and nullifies the endogenously generated radicals in various metabolic reactions in virtually all the aerobic living systems.

$$GSSG + NADPH + H^+ \quad \rightarrow \quad 2\,GSH + NADP^+$$

ANTIOXIDANTS:

Antioxidants are substances that are able to prevent or retard oxidation of lipid, proteins and DNA, and to protect the compounds or tissues from damage caused by oxygen or free radicals. Antioxidants are key line of defense capable of trapping free radicals by preventing radical formation, intercepting radicals from further damage to the body (Cotgreave *et al.*, 1988). Antioxidants also protect against glycation-derived free radicals and may have therapeutic potentials.

Apart from endogenous antioxidants a vast number of dietary agents also act as antioxidants. Currently available synthetic antioxidants like butylated hydroxyanisole, butylated hydroxytoluene, tertiary butyl-hydroquinone, propylgallate and gallic acid esters are known to ameliorate oxidative damages but they are suspected to prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants (Amarowicz *et al.*, 2000; Ghafar et al., 2010). Research on bioactive principles of essential oils extracted from various herbs and spices has become increasingly popular because essential oils have been discovered to have many functional properties such as antimicrobial, antioxidant and anticancer activities (Leal *et al.*, 2003; Lee and Shibamoto, 2002; Vardar-Unlu *et al.*, 2003). Plants, including herbs and spices, have many phytochemicals which are potential sources of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz et al., 2006). Green tea is considered a rich source of phenolic compounds and its consumption is considered to be a factor in the lower incidence of coronary heart disease in the Chinese population

(Zhang *et al.*, 2008). Similarly, parsley oil and two of its inherent bioactive phenolic compounds (i.e., myristicin and apiol) possess antioxidant activity (Zhang *et al.*, 2006). Therefore, polyphenolic compounds' health promoting effects reduce the risk of various diseases (Manach *et al.*, 2004) and inhibition of growth of pathogenic bacteria (Giroux *et al.*, 2001) which are often associated with the termination of free radical propagation in biological systems. Thus antioxidant capacity is widely used as a parameter to characterize medicinal plants and their bioactive components. There is growing interest in natural products with combined anti-glycation and antioxidant properties as they may have reduced toxicity.

PHYTOCHEMICALS:

Aromatic herbs and spices have been used for a long time in alternative medicine, not only to improve or modify the flavor of foods, but also to avoid its deterioration.

***Thymoquinone* (TQ)** (2-isopropyl-5-methyl-1,4-benzoquinone), is the main bioactive component of the volatile oil of *N. sativa* (Fig. 11). It has been used as antioxidant, anti-inflammatory and antineoplastic medicines for more than 2000 years (Hosseinzadeh and Parvardeh, 2004; Trang *et al.*, 1993). Generally *Nigella sativa* seeds contain more than 30% fixed oil and 0.40% to 0.45% volatile oil. TQ represents 18.4 to 24% of the *N. sativa* volatile oil (Arslan et al., 2005). TQ can also be found in other plants such as Callitris quadrivalvis, Monarda fistulosa, Juniperus cedrus, Tetraclinis articulata, and Nepeta leucophylla. Quinones are ubiquitous in nature and constitute an important class of naturally occurring compounds found in plants, fungi, and bacteria. Current human exposure to quinones occurs via the diet as well as clinically. Benzoquinones are potentially derivable by oxidation of suitable phenolic compounds. Many of these benzoquinones have important biochemical functions in electron transport systems for respiration or photosynthesis (Dewick, 2001). The pharmacological properties attributed to naturally occurring quinones are thus well established. For example, thymoquinone presents anticonvulsant activity in the petit mal epilepsy (Hosseinzadeh and Parvardeh, 2004). *N. sativa* has been traditionally used as a natural remedy for a number of illnesses and conditions such as diabetes, inflammation, bronchitis, fever, and influenza (Ali and Blunden, 2003). The antioxidant effect of TQ is associated with its potential to alter

"redox state" and its scavenging activity against free radicals through modulation of hepatic and extra-hepatic antioxidant enzymes (Karaman et al., 2010). TQ protects the kidney against ifosfamide, mercuric chloride, cisplatin and doxorubicin-induced damage by preventing renal GSH depletion and anti-lipid peroxidation product accumulation, thereby improving renal functioning (Badary, 1999; Badary *et al.*, 1997; Fouda *et al.*, 2008). Khattab and Nagi (2007) assessed the protective effects of TQ after chronic inhibition of nitric oxide synthesis with N (omega)-nitro-l-arginine methyl esters and found that treatment with TQ increased GSH to normal levels and inhibited the *in-vitro* production of superoxide radicals.

***Thymol* (TL)** (2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, isomeric with carvacrol, found in oil of thyme (Fig. 12) and has been commonly used in foods mainly for flavor, aroma and preservation and also in folk medicine since the ancient Greeks, Egyptians and Romans (Baser, 1993; Baser, 1994). TL can be used for the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria (Didry *et al.*, 1994; Kohlert *et al.*, 2002). Other plants that contain thymol are Origanum compactum and Thymus glandulosus (Tai *et al.*, 2002).

***Eugenol* (EU)** (4-allyl-2-methoxyphenol) is a methoxyphenol compound having a short hydrocarbon chain in its structure (Gulcin, 2011). It is found in virtually all spices but bay leaves and cloves are considered the best sources of it (Fig. 13) (Tai *et al.*, 2002). It is one of the major components constituting about 80-95% of clove oil (Szabadics and Erdelyi, 2000). Pharmacologic studies have demonstrated that EU has anticonvulsant (Dallmeier and Carlini, 1981), local anesthetic (Brodin and Roed, 1984), antistress (Sen *et al.*, 1992), bacteriostatic and bactericidal (Walsh *et al.*, 2003) and antifungal properties (Lee *et al.*, 2007). EU and its isoform, isoeugenol have been documented to be potential inhibitors of copper-dependent oxidation of LDL (Ito *et al.*, 2005). Besides, treatment of EU with fast decaying fruits like strawberries increased their average shelf life and also preserved their nutrient values of sugar and organic acids. The treatment also increased the content of total phenolics, anthocyanins and flavonoids (Wang *et al.*, 2007).

Fig. 11: (A) *Nigella sativa* seeds and flower, (B) Chemical structure of Thymoquinone (component of *N. sativa*).

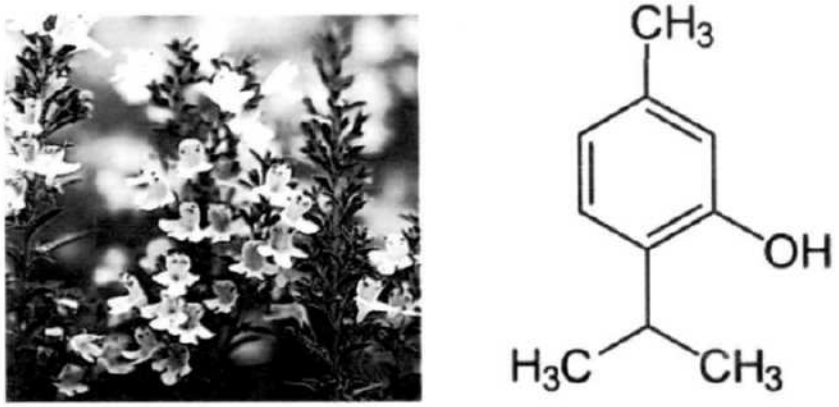

Fig. 12: (A) *Thymus vulgaris* plant, (B) Chemical structure of thymol (component of *Thymus vulgaris*)

Fig. 13: (A) *Eugenia caryophyllata* plant, (B) Chemical structure of Eugenol (component of *Eugenia caryophyllata*).

OBJECTIVE OF THE PRESENT STUDY:

Glycation is the sequence of non-enzymatic reactions involving interaction between reducing sugars and the nucleophilic groups of proteins and other biomolecules. It is ubiquitous in nature and occurs in the cells of all living organisms, albeit at a very slow rate. The rate of glycation however increases remarkably during hyperglycemia, in diabetes and related disorders. Persistent hyperglycemia induces abnormal changes such as increase of AGEs formation, increase of polyol pathway flux, and activation of protein kinase C isoforms. Glycation is also accompanied by the formation of highly reactive and damaging ROS. Free radicals and glycation end products are known to cause severe protein damage resulting in major structural alterations and loss of biological function.

A large body of evidences indicate that glycation is a key molecular basis of diabetic complications. Hyperglycemia is regarded as the primary cause of diabetic microvascular complications that eventually contribute to diabetic macrovascular disease. Diabetic complications usually arise as a result of non-enzymatic protein glycation which leads to the formation of heterogenous, toxic, and antigenic AGEs. Three routes have been proposed for AGEs formation: (i) autoxidative pathway (sugar gives reactive products by autoxidation), (ii) conventional Amadori rearrangement, and (iii) Schiff base formation. Reactive oxygen species (ROS) such as $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$ contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process includes also oxidative steps and is therefore called glycoxidation. Regarding the significance of glycoxidative stress to diabetic pathology, phytochemicals that interfere with glycation reactions may be beneficial in restricting the complications accompanying diabetes and related disorders. Restriction/prevention of glycation and oxidative stress are therefore mooted as an effective strategy for alleviation of complications associated with hyperglycemia. These days, scientific interests in the functional components of food, with health protecting potencies, are increasing mainly due to their various biological activities and low cytotoxic effects.

Since glycation- and oxidation-induced biochemical changes are known to participate in the overall diabetic complications, the present study was planned to determine antioxidant activity of TQ, TL and EU and their protective effect against AAPH-induced RBC hemolysis. The study was also extended to determine the possibility of preventing

the glucose-induced modification in HSA, as it being the most abundant protein in human blood, by these phytochemicals in concentration and time dependent manner. Furthermore, increase in glycoxidation products in plasma and tissue proteins suggest that oxidative stress increases in diabetes. Hence, the present study was further extended *ex vivo* to evaluate the serum level of lipid peroxides, protein carbonyls, total antioxidant capacity and electrophoretic pattern of albumin in healthy and diabetic patients. The protective effect of TQ, TL and EU on these parameters was also determined. For this, diabetic serum samples were incubated in absence and presence of TQ-2 (30 μM), TL-2 (30 μM) or EU-2 (0.6 μM) for 21 days at 37°C.

Materials and Methods

Chemicals

Human serum albumin, thymoquinone, thymol, eugenol, aminoguanidine hydrochloride, diethylene triamine penta-acetic acid (DTPA), bovine serum albumin, sodium azide, superoxide dismutase, cytochrome c, dialysis tubing, agarose, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Company, USA. D-glucose, 2,4-dinitrophenyl hydrazine (DNPH), ethanol, sodium carbonate and formaldehyde were purchased from Merck, India. Acrylamide, bis-acrylamide, ammonium persulphate and N,N,N',N'- tetramethyl ethylene diamine (TEMED) were from Bio-Rad Laboratories, USA. Nitroblue tetrazolium (NBT), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), sulphosalicylic acid, 2,4,6-trinitrobenzene sulphonic acid (TNBS), Folin-Ciocalteau reagent, bromophenol blue, gallic acid, ascorbic acid (AA) and sodium dodecyl sulphate were from Sisco Research Laboratories, India. Polystyrene microtitre flat bottom ELISA plates and modules were purchased from NUNC, Denmark. Nitrocellulose syringe filters were purchased from AXIVA Sichem Biotech, India. Silver nitrate, trichloroacetic acid, methanol, sodium chloride, sodium acetate, isopropanol, glacial acetic acid, glycine, EDTA, ethylacetate, potassium ferricyanide, ferric chloride, copper sulphate, sodium potassium tartarate and sodium hydroxide pellets were obtained from Qualigens Fine Chemicals, India. All other chemicals and reagents were of the highest analytical grade available.

Equipments

Spectrophotometer (model, U-2910; Hitachi, Japan), Spectrofluorometer (model, RF-5301; Shimadzu, Japan), Spectropolarimeter (model, JASCO J-815; USA), Centrifuge (model, 3K30; Sigma, Germany), Polyacrylamide gel electrophoresis assembly (Genei, India), Densitometer (model, GS-800; Bio-Rad, USA), ELISA reader (Labsystem Multiskan, Finland), pH meter (model, L1-120; ELICO, India), Gel-doc (Bio-Rad Laboratories, USA) and FTIR spectrophotometer (model, 8300; Shimadzu, Japan) were the major equipments used in this study.

Collection of blood samples

The protocol used for the study was in accordance with guidelines of institutional ethical committee. Forty healthy and forty type 2 diabetic patients diagnosed using World Health Organization criteria were included in the study. Fasting venous blood for estimation of MDA, protein carbonyl, FRAP and glutathione content were drawn after obtaining verbal consent from type 2 diabetic patients attending outpatient department (OPD) clinics at Rajiv Gandhi Centre for Diabetes and Endocrinology, Jawaharlal Nehru Medical College Hospital, Aligarh Muslim University, India. It was ensured that none of the diabetic patients were suffering from other autoimmune diseases. Normal human blood was obtained from age- and sex- matched healthy individuals (Table 1). All blood samples were collected in plain vials and left for clot formation. Serum was separated and stored in small aliquots at -20°C.

Table 1: Clinical features and demographic profile of healthy and diabetic subjects.

Variables	**Healthy controls (Mean ± SD)**	**Type 2 diabetes (Mean ± SD)**
Sample size (n)	40	40
Age (years)	49.9 ± 5.71	51.6 ± 6.91
Gender (male/female)	18/22	21/39
BMI (Kg/m^2)	22.3 ± 1.39	28.7 ± 1.6
Fasting glucose (mg/dl)	89 ± 3.14	167 ± 23.38
Post prandial glucose (mg/dl)	126 ± 12.34	248 ± 23.36
HbA_{1c}	4.8 ± 0.43	7.46 ± 0.54

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Activity

The free radical scavenging activity of TQ, TL, EU and standard reference compound *i.e*, Gallic acid (GA) was analyzed by the DPPH assay as described by Sanchez-Moreno *et al.*, (1998) with minor modification. In this assay, 1 ml of varying concentrations of TQ (0.25-2.0 mg/ml), TL (0.25-2.0 mg/ml) and EU (1-60 µg/ml) dissolved in 1 ml of ethanol, were mixed with 1 ml of ethanol solution of DPPH (0.2 mM). The mixture was vortexed and incubated for 30 min. The optical density of the solution was measured at 517 nm using Hitachi, U-2910 spectrophotometer. Gallic acid (µg/ml) has been used as a standard. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{[A_{DPPH} - A_{TEST}]}{A_{DPPH}} \times 100$$

Where A_{DPPH} is the absorbance of DPPH without test compound (TQ, TL, EU and GA), and A_{TEST} is the absorbance of DPPH in the presence of test compound.

Reducing Power

Total reducing power was determined as described by Zhu *et al.*, (2004) with some modifications. TQ (0.5-2.0 mg/ml), TL (0.5-2.0 mg/ml) and EU (2.5-10 µg/ml) in 1 ml of ethanol were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]; the mixture was then incubated at 50°C for 30 minute. 2.5 ml of trichloroacetic acid (10%) was then added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increase in absorbance indicates increased reducing power of the phytochemical.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay is considered as a very reliable method to assess the total reducing potential of any biological active compound or extract. In the present study, it was carried out by the method of Benzie and Strain (1996) with slight modifications. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous

(Fe^{2+}-TPTZ), intensive blue colored form in the presence of antioxidant. 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM $FeCl_3$ were mixed in a ratio of 10:1:1 to be a working FRAP reagent. 100 µl of TQ, TL and EU were mixed with 3 ml of FRAP reagent and incubated at 37°C for 30 min. The absorbance at 593 nm was monitored. All reagents were freshly prepared before use.

Ferric reducing antioxidant power of healthy, diabetic and phytochemical treated diabetic serum samples was also determined by the same above protocol. Diabetic serum samples were incubated with three concentrations of TQ, TL and EU for 1 hour at 37°C. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM and 6.0 µM. 20 µl of each phytochemical was incubated in 80 µl of serum of diabetic patients. 80 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. 3 ml of FRAP reagent was added to each tube and incubated at 37°C for 30 min. The absorbance at 593 nm was monitored. Aqueous solution of known Fe (II) concentration was used for calibration (in a range of 100-1000 µmol/l) (Table 2, Fig. 14).

Blank: FRAP reagent.

Table 2 Standards: 1mM standard solution of ferrous sulphate was prepared by mixing 0.278 g of $FeSO_4.7H_2O$ in 1 litre distilled water. They were diluted as follows to make a series of standards of different molarities.

Standard concentration (mM)	**FeSO4.7H2O solution (ml)**	**Distilled water (ml)**
0.1	1	9
0.2	2	8
0.4	4	6
0.6	6	4
0.8	8	2
1.0	10	0

Freeze at -20° C in 0.2 ml aliquots in ependorfs.

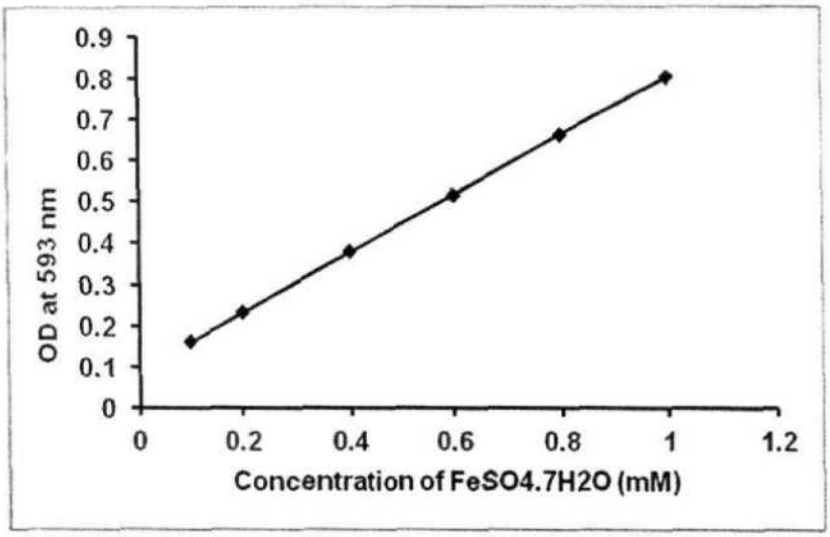

Fig. 14: Standard curve for ferrous sulphate for the determination of FRAP equivalent.

Metal Chelating Activity

The chelation of ferrous ions by TQ, TL and EU was estimated by the method of Dinis *et al.,* 1994 with slight modifications. Different concentrations of the TQ (12.5-200 µg/ml), TL (12.5-200 µg/ml), EU (3.125-25 µg/ml) and AA (3.125-25 µg/ml) were added to a solution of 1 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 1 mM ferrozine (0.1 ml) and the mixture was finally quantified to 1 ml with methanol, shaken vigorously, and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm.

AAPH-induced RBC hemolysis assay

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J. N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered saline (PBS), pH 7.4. During every wash, RBCs were centrifuged at 4000 rpm for 10 min to obtain packed cell preparation (Miki *et al.,* 1987). The packed RBC was suspended in four volumes of PBS solution after the last wash. AAPH, a peroxyl radical initiator, was used for RBC hemolysis. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and

proteins, resulting in hemolysis. 0.5 ml of the erythrocyte suspension was mixed with 0.5 ml of PBS solution containing varying amounts of TQ, TL and EU and to this 0.5 ml of 200 mM AAPH was added. The reaction mixture was shaken gently while being incubated at 37°C for 3 hours. After incubation, reaction mixture was diluted with eight volumes of PBS and centrifuged at 4000 rpm for 5 min. The Absorbance (A) of the supernatant was recorded at 540 nm. Percent inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{[A_{AAPH} \; A_{TEST}]}{A_{AAPH}} \times 100$$

Where A_{AAPH} is the absorbance of AAPH at 540 nm and A_{TEST} is the absorbance of TQ, TL, EU and AA at 540 nm.

Estimation of malondialdehyde (MDA) level

Free radicals, by their unstable and transient nature are difficult to measure directly. Their tendency to cause lipid peroxidation has been used as an indirect measure. Hence, estimation of lipid peroxides (markers of lipid peroxidation) was done by measuring MDA (malondialdehyde), which is a stable end byproduct of lipid peroxidation. MDA is one of the most reliable products to assess the extent of lipid peroxidation. Its level was determined by the procedure of Ohkawa *et al.*, (1979) with some modifications. It is based on reaction of Thiobarbituric acid (TBA) with MDA. One molecule of MDA reacts stoichiometrically with two molecules of TBA at pH 3.5. The pink color chromogen can be measured spectrophotometrically at 532 nm with the production of a pink pigment having an absorbance maximum at 532 nm.

Preparation of TBA reagent

TBA reagent was prepared by mixing 0.1 ml SDS (8.1% w/v), 0.75 ml acetic acid (20% v/v, pH adjusted to 3.5 with 5N NaOH) and 0.75 ml TBA (0.8% w/v).

Procedure

Three concentrations of thymoquinone, thymol and eugenol were prepared for *in-situ* study. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM and 6.0 µM. 20 µl of each phytochemical was incubated in 100 µl of serum of diabetic patients for 1 hour at 37°C. 100 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. Then 1.6 ml of TBA reagent was added to all incubated tubes. After vortexing, samples were incubated for 1 hour in 95°C and after cooling with tap water centrifuged at 4,000 rpm for 10 min. Supernatant was separated and measured spectrophotometrically at 532 nm. TBARS values were expressed as MDA equivalents. Concentration was calculated using extinction coefficient of the TBA reagent = $1.56 \times 10^5 mol^{-1}cm^{-1}$. MDA concentration is expressed in terms of nmoles/ml of serum.

Determination of Reduced glutathione (GSH) content

Reduced glutathione (GSH) level was determined by method of Ellman, (1959) with slight modifications. 500 µl of serum was precipitated with 500 µl sulphosalicylic acid (4%). The samples were then centrifuged at 1,200 rpm for 10 min at 4°C. 400 µl of supernatant was taken and 400 µl of DTNB (3 mM) and 2.2 ml of phosphate buffer (0.2 N, pH 8.9) was added. The yellow color developed was read at 412 nm and the amount of reduced glutathione was calculated using the molar extinction coefficient value of $1.36 \times 10^4 cm^{-1} mol^{-1}$.

MODIFICATION OF HUMAN SERUM ALBUMIN BY GLUCOSE:

Commercial HSA that gave single band in SDS-PAGE was used without purification. Lyophilized HSA was reconstituted with 20 mM phosphate buffer (pH 7.4) to make a stock solution of 3 mg/ml and stored at -20°C. In order to induce glycation, the protocol used by Miyazawa *et al.*, (1998) was adopted with slight modifications. HSA 1 mg/ml (15.15 µM), was incubated for 7, 14, 21, and 28 days at 37°C in dark with 100 mM glucose in 20 mM phosphate buffer (pH 7.4). The reaction mixtures were pre-filtered

through a nitrocellulose filter 0.2 μm pore-sizes in pre-autoclaved boxes in order to maintain sterile conditions during incubation. Samples incubated without sugar similarly served as control. After incubation, samples were extensively dialyzed against PBS in order to remove excess of sugars and stored at -20°C for further analysis. Protein concentration was measured by Lowry *et al.*, (1951).

IN-VITRO GLYCATION OF HSA WITH GLUCOSE ALONG WITH VARIOUS COMPOUNDS:

HSA was incubated with 1mM aminoguanidine (standard anti-glycating agent) in the presence of 100 mM glucose. HSA was also incubated with varying concentrations of thymoquinone (3, 30 and 300 μM), thymol (3, 30 and 300 μM) and eugenol (0.06, 0.6 and 6.0 μM) in presence of 100 mM glucose. They are incubated under similar conditions for 4 weeks. HSA incubated without sugar served as control. Before further analysis each sample was extensively dialyzed against the buffer in order to remove excess of sugars.

UV-visible Spectrophotometry

The ultraviolet absorption spectra of native, glycated and phytochemical treated glycated HSA samples were recorded in the wavelength range 200–400 nm on a Hitachi U-2910 spectrophotometer, using a cuvette of 1 cm pathlength. One milligram of the sample in a total volume of 3 ml was taken for spectral analysis.

Fluorescence spectroscopy

Native, glycated and phytochemical treated samples were analyzed by measuring intrinsic fluorescence at 25°C on Shimadzu (RF 5301-PC) spectrofluorometer. The fluorescence of tryptophan residue (Tryptophan-214) in all samples was monitored with excitation at 285 nm and emission range was taken at 290 – 400 nm (Shaklai *et al.*, 1984). The concentration of protein samples was taken as 2 μM.

AGE- specific Fluorescence

AGEs formation were measured by determining the fluorescence by excitation at 370 nm and emission between 400 - 500 nm (Lapolla, 1992) using Shimadzu (RF 5301-PC)

spectrofluorometer. Loss of fluorescence intensity (FI) was calculated using the following equation-

$$\% \text{ Loss of FI} = \frac{[FI_{\text{native HSA}} - FI_{\text{glycated HSA}}]}{FI_{\text{native HSA}}} \times 100$$

Circular dichroism (CD) studies

CD spectra were recorded on JASCO spectropolarimeter (J-815) calibrated with D-10-camphorsulfonic acid. The measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab's RTE 110 water bath with a temperature accuracy of ± 0.1°C. Far-UV CD was used to measure the changes in the secondary structure of HSA (3.0 µM) in 20 mM phosphate buffer (pH 7.4). Protein samples were placed in cylindrical quartz cuvettes of pathlength 1mm. Each spectrum was the result of average of four scans. CD measurements were performed on HSA samples withdrawn every week from the reaction mixture under incubation for 4 weeks kept at 37°C. The results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ which is defined as:

$$\text{MRE} = \frac{\theta_{\text{observed}}\,(\text{mdeg})}{10 \times (\text{n}) \times C_p \times l}$$

Where, $\theta_{observed}$ is the CD in milli-degree, n is the number of amino acid residues (585 – 1 = 584), l is the path length of the cell, and C_p is the concentration of protein in moles/litre. Helical content was calculated from the MRE values at 222 nm using the following equation (Chen *et al.*, 1972).

$$\% \text{ α-helix} = \frac{[MRE_{222\,\text{nm}} - 2340]}{30300} \times 100$$

Fourier Transform Infra-red spectroscopy (FTIR)

To confirm the interaction between HSA, aminoguanidine and phytochemicals (TQ, TL and EU) in absence and presence of glucose, FTIR was conducted. FTIR spectra of native, glycated and phytochemical treated samples were recorded on Shimadzu-8300 FTIR spectrophotometer (Tokyo, Japan) in the spectral range of 400 – 4000 cm^{-1}. Samples to be analyzed were loaded between two potassium bromide discs by hydraulic pressing.

Determination of Protein Concentration

The protein content in all the samples was estimated by the method of Lowry *et al.*, (1951). The protein estimation by this method utilizes alkali (to keep the pH high), Cu^{+2} ions (to chelate proteins) and tartarate (to keep the Cu^{+2} ions in solution at high pH).

Folin-Ciocalteau reagent

The Folin-Ciocalteau reagent was diluted 1:1 with distilled water before use.

Alkaline copper reagent

The components of alkali copper reagent were prepared as follows:

- 2% sodium carbonate in 100 mM sodium hydroxide
- 0.5% copper sulphate in 1.0% sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

Procedure

Aliquots of protein solution were taken in separate tubes and the final volume was made up to 1 ml. To this, 5 ml of alkaline copper reagent was added. This solution was kept as such at room temperature for 10 minutes followed by addition of 1 ml Folin-Ciocalteau reagent. The test tubes were vortexed followed by incubation for 30 minutes at room temperature. The resulting blue color intensity was read at 660 nm against the reagent blank using spectrophotometer. A standard curve was prepared using BSA as standard (Fig. 15).

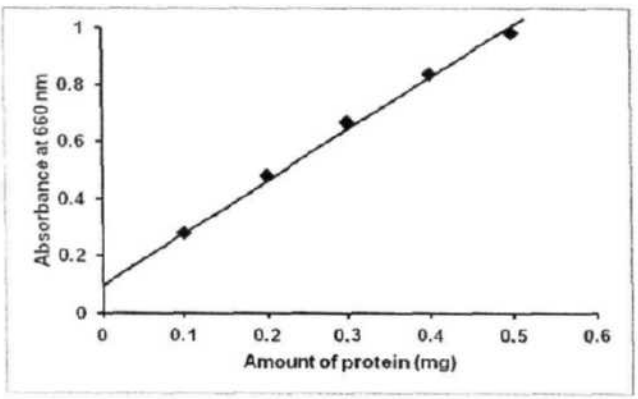

Fig. 15: Bovine serum albumin (BSA) standard curve using Lowry's method of protein estimation.

Ketoamine estimation by Nitrobluetetrazolium assay

Glycation of HSA or level of Amadori product was determined by an established colorimetric procedure using NBT (Mashiba *et al.,* 1992) with slight modifications. Native, glycated and phytochemical treated HSA samples (50 µl) were added to the wells of 96-well microtitre plate in duplicate. One hundred µl of NBT reagent (250 µM in 0.1 M carbonate buffer, pH 10.35) was added to each well and incubated at 37°C for 2 hours. The plate was read in a microplate reader at 550 nm. The amount of ketoamine in all samples was determined using 12640 $cm^{-1}M^{-1}$ as molar extinction coefficient of monoformazan.

Determination of Protein bound carbonyl groups

Carbonyl content of native, glycated HSA and phytochemical treated glycated HSA was estimated by a published procedure of Levine *et al.,* (1990) with slight modifications. Briefly, 200 µl aliquot (containing 0.1 mg of protein) was mixed with 400 µl of 7 mM dinitrophenyl hydrazine (DNPH) in 2M HCl. The mixtures were run in duplicate and the control protein samples were devoid of DNPH. After incubation for 1 hour at room temperature, 500µl trichloroacetic acid (4% w/v) was added to precipitate DNP-hydrazones. Mixture was centrifuged for 5 min at 14,000 rpm. The pellet was dispersed in 1 ml ethanol-ethylacetate (1:1 v/v), in order to remove unreacted DNPH and centrifuged. After 3 such washes, the pellet was dissolved in 0.6 ml of 6M guanidinium hydrochloride solution in 20 mM phosphate buffer already adjusted to pH 2.3 with trifluoroacetic acid. The hydrazones were dissolved completely only by overnight freezing at -20°C and thawing. From the solution, 200 µl aliquot was taken into a microplate and read at 379 nm by a microplate reader. The results were expressed as the number of nmoles of carbonyl per mg of sample protein using a molar absorption coefficient 22,000 $M^{-1}cm^{-1}$. Samples were analyzed against a blank of 1 ml of 6 M guanidinium hydrochloride solution.

Similar procedure was used for the estimation of protein carbonyl contents in the serum of healthy and diabetic patients in case of *ex vivo* studies. Three concentrations of thymoquinone, thymol and eugenol were prepared for *ex vivo* study. The concentrations of TQ and TL were 3 µM, 30 µM and 300 µM while those for EU were 0.06 µM, 0.6 µM

and 6.0 µM. 100 µl of diabetic serum was incubated with 20 µl of each phytochemical for 1 hour at 37°C. 100 µl of healthy and diabetic serum samples incubated with 20 µl of 20 mM phosphate buffer (pH 7.4) served as control. After vortexing, all serum samples followed the Levine *et al.*, (1990) protocol.

Determination of Free Amino Groups

The free amino groups of native, glycated and compound treated glycated HSA were measured using TNBS procedure described by Haynes *et al.*, (1967). To 0.2 ml of glycated protein, 1.8 ml of sodium tetraborate buffer pH 9.3 was added. Further 20 µl of 0.13 M TNBS solution was added. The reaction mixture thus formed was incubated at 37°C for 30 min. The absorbance of the solution was read at 420 nm against a blank.

Detection of superoxide anion radicals in glycated HSA in presence of phytochemicals

Superoxide radical ($O^{\bullet -}$) was measured by the cytochrome c reduction method (Beauchamp and Fridovich, 1971). The effect of AG (1 mM) and varying concentrations of TQ (3, 30 and 300 µM), TL (3, 30 and 300 µM) and EU (0.06, 0.6 and 6.0 µM) on the generation of superoxide radicals during glycation of HSA was determined. The reaction tube contained HSA (15.15 µM), glucose (100 mM), DTPA (0.01 mM), cytochrome c (200 µM) in absence and presence of AG, TQ, TL or EU. Reaction was carried out at 37°C for 20 min in 100 mM phosphate buffer (pH 7.4). The reduction rate was monitored at 550 nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed as described earlier by Laemmli, (1970) using slab gel electrophoresis apparatus. The following stock solutions were prepared:

Acrylamide-Bisacrylamide(30:0.8)

A stock solution was prepared by dissolving 30 g of acrylamide and 0.8 mg of bisacrylamide in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4°C in an amber color bottle.

Resolving Gel Buffer

A stock solution was prepared by dissolving 9.08 g Tris base in 40 ml distilled water. The pH was adjusted to 8.8 and the final volume brought to 50 ml with distilled water.

Electrode buffer

3 g Tris base, 14.4 g glycine and 1.0 g SDS were dissolved in distilled water, pH adjusted to 8.3 and final volume was made to 1 litre.

Procedure for PAGE

Thoroughly cleaned glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from sides and bottom. The resolving gel mixture (10%) was prepared by mixing the components listed in table 3 and poured between the glass plates and allowed to polymerize at room temperature. Protein samples mixed with one fourth of sample dye (10% SDS, 50% glycerol, 1M Tris, pH 6.8, 5% β-mercaptoethanol and 1% bromophenol blue as a tracking dye) were boiled at 100°C for 5 minutes and then loaded into the wells, and electrophoresis was carried out at 80 volts for 3-4 hours in electrode buffer.

For *ex vivo* studies, diabetic serum samples were incubated in absence and presence of TQ-2 (30 μM), TL-2 (30 μM) or EU-2 (0.6 μM) for 14 days at 37°C. Effect was also compared with HSA incubated in same conditions in presence of 20 mM glucose to check the effect on glycation reaction in excess glucose and the preventive/ inhibitory effect by the phytochemicals.

Table-3: Recipe for 10% resolving gel

Component	**Volume**
Distilled water	4.0 ml
Acrylamide-bisacrylamide (30: 0.8)	3.3 ml
Resolving gel buffer	2.5 ml
10% SDS	0.1 ml
1.5% ammonium persulphate	0.1 ml
TEMED	4.0 μl

Serum of healthy individuals without any treatment served as control. They were analyzed by 10% SDS-PAGE in the presence of thiol-reductant β-mercaptoethanol followed by silver staining.

Silver Staining

The procedure described by Merril *et al.*, (1982) was followed. After electrophoresis the protein bands were fixed by rapidly immersing in a mixture of 40% methanol and 13.5% formaldehyde for 10 min with instant shaking. The gel was washed with distilled water twice at an interval of 5 min. Then the gel was immersed in 0.02% sodium thiosulphate solution for 2 min. The gel was again rinsed twice with distilled water at an interval of 20 second. This was followed by incubation with 0.1% silver nitrate solution for 10 min. The gel was rinsed with distilled water briefly, immersed in developer solution (3% sodium carbonate solution containing 50 µl formaldehyde and 2 ml of 0.02% sodium thiosulphate) for 15 min or until properly stained. The reaction was stopped by transferring the gel to stopper solution (25% isopropanol solution containing 10% acetic acid glacial). The gel was washed twice with distilled water and finally stored in distilled water.

Densitometric Analysis

Image analysis of gels was performed to show the inhibition of cross-linked AGEs and/or fragmentation in different concentrations of TQ, TL and EU. Since protein concentration loaded in each well of gel remains same throughout the whole study i.e. 10 µg, therefore, the area selected for performing densitometry of each band was taken to be same or equal. Bands were compared within the same gel and sufficient background was included.

Results

DPPH radical scavenging effects

The scavenging effects of TQ, TL and EU on DPPH radicals are shown in Fig. 16. All the phytochemicals scavenged DPPH in a dose-dependent manner. As seen in Fig. 16 (a), TQ showed maximum DPPH radicals scavenging activity up to 78.13% at 2.0 mg/ml concentration while it was 82.26% at same concentration for TL (Fig. 16 b) and 88.4% for EU at much lower concentration (60 µg/ml) (Fig.16 c). In a similar assay, gallic acid (GA) as standard reference compound scavenged up to 94.67% at 18 µg/ml, having EC_{50} value of 2.87 µg/ml (Fig. 16 d). EC_{50} is the concentration of a drug/ antioxidant that gives half-maximal response. EC_{50} value of TQ, TL and EU are 1.07 ± 0.03 mg/ml, 393.3 ± 11.54 µg/ml and 10.07 ± 1.9 µg/ml respectively. Among the three phytochemicals examined, EU exhibited the strongest efficiency and showed over 50% scavenging effect of DPPH at a concentration of 10.07 µg/ml. Therefore, EU had the highest hydrogen-donating capacity, followed by TL, while TQ rendered the weakest effect. However, the EC_{50} value of TQ, TL and EU was higher than GA revealing that these phytochemicals had lesser free radical scavenging potential than GA.

Reduction Potential

Total reducing power of TQ, TL and EU was measured by method of Zhu *et al.*, (2004) that involves reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$ leading to formation of Perl's Prussian blue complex determined at 700 nm. Ascorbic acid (AA) was used as standard. Fig. 17, shows the dose response curves for the reducing powers of TQ, TL and EU. As reflected in Fig. 17(c) and (d), the reducing power of EU was maximum (0.119) at 10 µg/ml concentration which is higher than standard AA (0.109) at 50 µg/ml. The reducing power of all phytochemicals increased with concentration and these varied in the order of TQ<TL<EU.

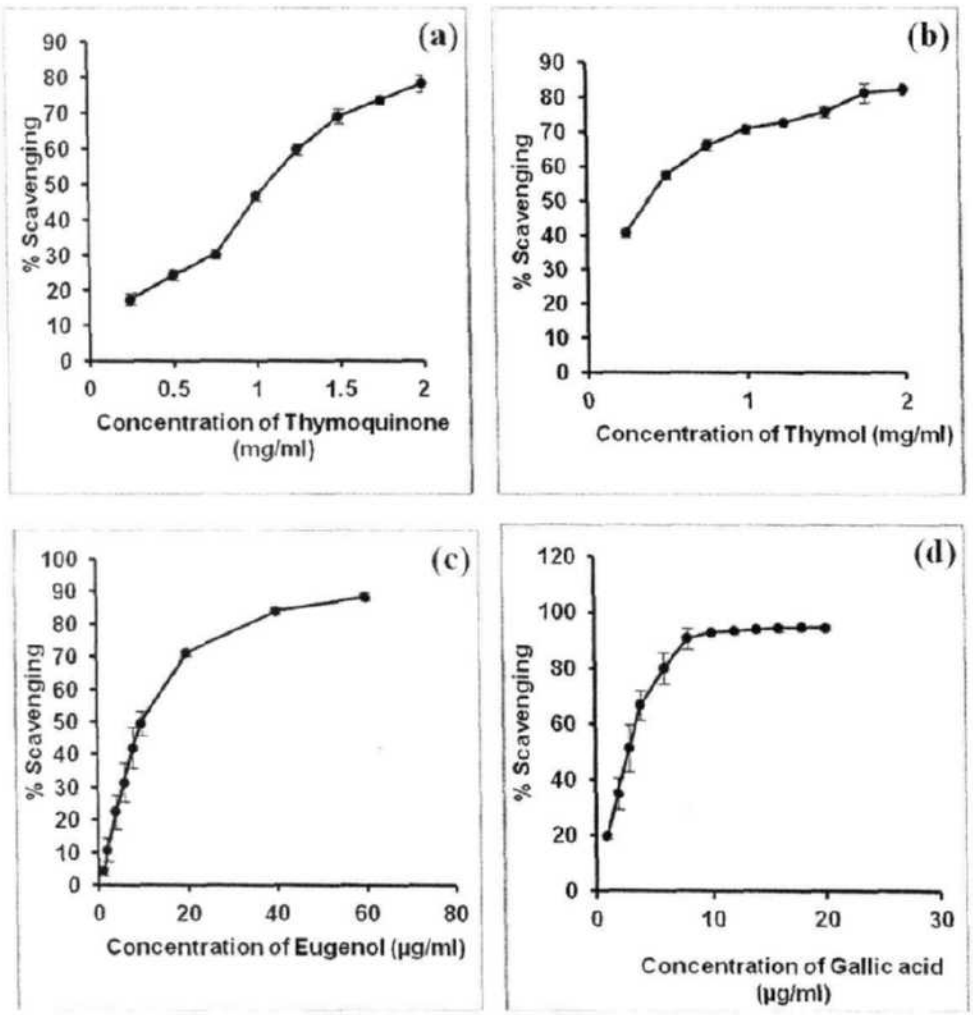

Fig. 16: Percent DPPH scavenging activities of (a) TQ, (b) TL, (c) EU and (d) GA. All the data has been represented as Mean ± SD of triplicate samples.

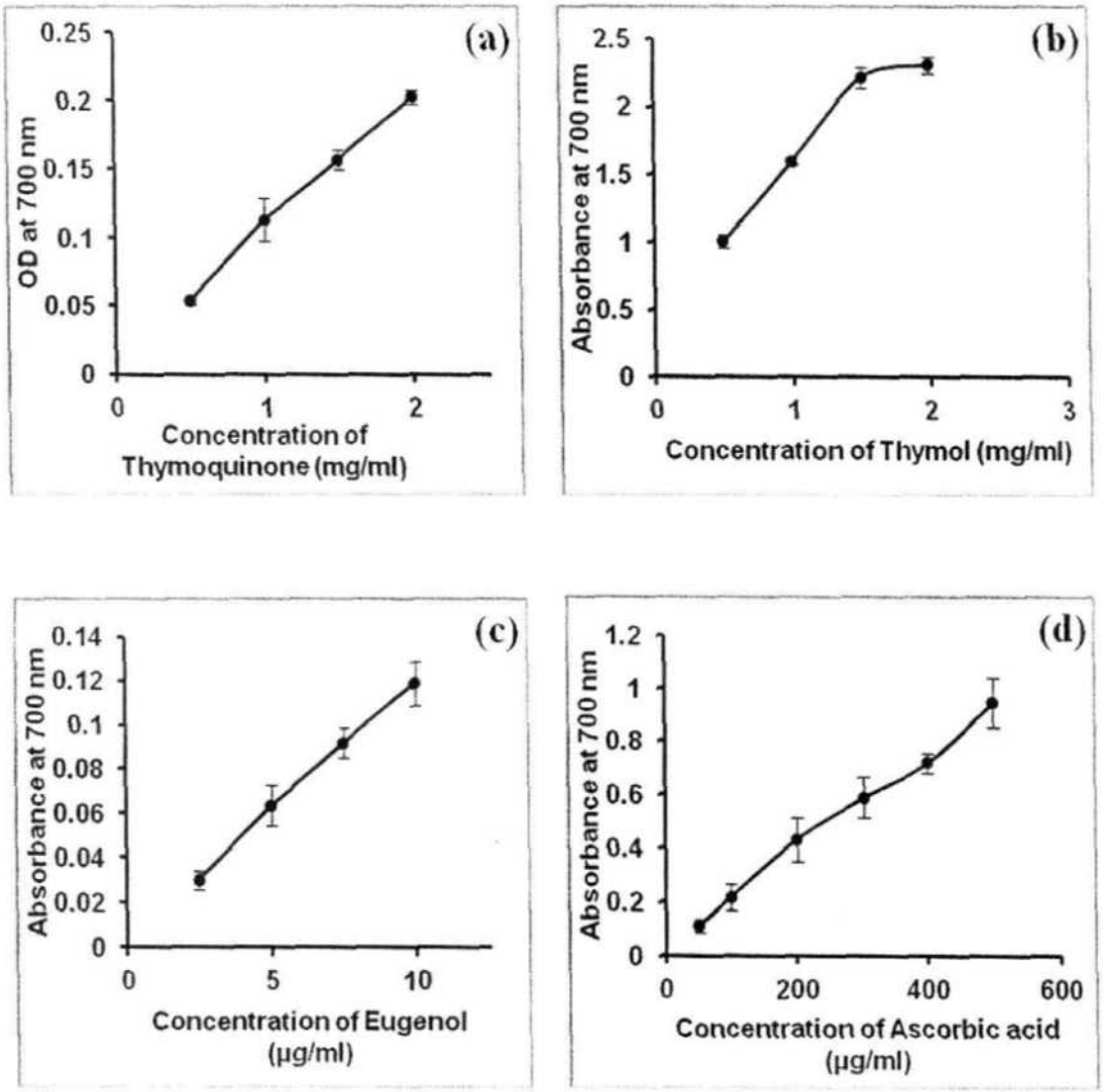

Fig. 17: Total Reducing ability at various concentrations of (a) TQ, (b) TL, (c) EU and (d) AA. All the data has been represented as Mean ± SD of triplicate samples.

Ferric reducing antioxidant power (FRAP) assay

Several assays have been introduced for the measurement of antioxidant activity of phytochemicals including total antioxidant capacity by FRAP assay. FRAP method was used to evaluate the reducing power of TQ, TL and EU. In this method the reduction of ferric-tripyridyltriazine complex to its ferrous colored form is evaluated in the presence of phytochemicals. The FRAP values have been calculated by comparing the absorbance change at 593 nm in test samples with those containing ferrous ions in known concentrations. FRAP value was calculated from the standard graph of ferrous sulphate (Standard curve equation: $Y= 0.71274x + 0.09092$; $r^2= 0.999$). As shown in Figure 18, FRAP value of TQ, TL and EU was found to be 2.73 ± 0.59, 25.31 ± 7.63 and 250.16 ± 18.92 mM Fe^{2+}/mg of phytochemical respectively.

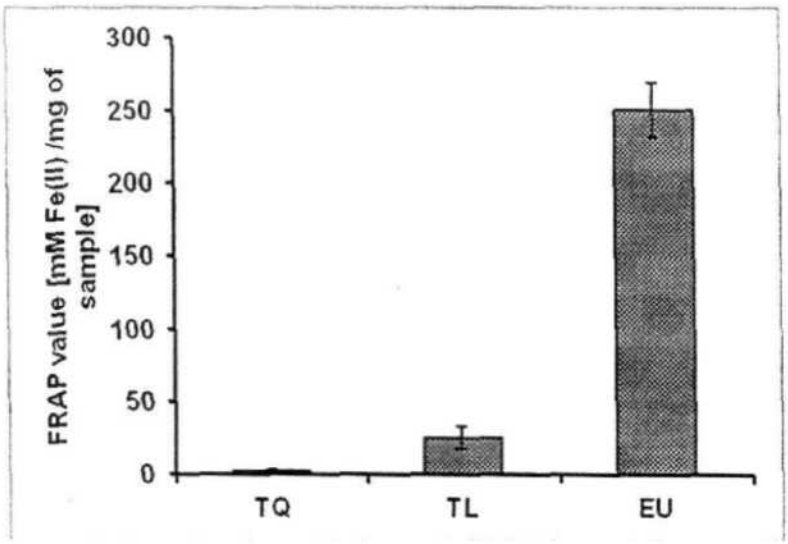

Fig. 18: Ferric reducing antioxidant power of TQ, TL and EU. All the data has been represented as Mean ± SD of triplicate samples.

Metal Chelating Activity

In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with consequent decrease in the intensity of the red color of the complex. As shown in Fig. 19, TQ, TL, EU and AA were capable of chelating ferrous ions up to 63.55%, 73.11%, 80.96% and 83.48% at the concentrations of 200, 200, 21.87 and 21.87 µg/ml respectively. The concentration at which TQ, TL, EU and AA showed 50% chelating activity was found to be 70, 40, 13.2 and 11.6 µg/ml respectively. Based on these data, it is clear that all the phytochemicals contained high antioxidant activity and chelating properties.

AAPH induced RBC hemolysis assay

In order to compare the antioxidative activity of phytochemicals quantitatively, it is essential that the relationship between concentration of phytochemicals and percent inhibition is established, in which the appropriate time of AAPH incubation is 3 hours in this case. The results showed inhibition of hemolysis in the presence of TQ, TL, EU and ascorbic acid (AA) in a dose dependent manner (Fig. 20). TQ, TL, EU and AA inhibited AAPH-induced hemolysis at concentrations ranging from 12.5 to 100 µg/ml, 12.5 to 100 µg/ml, 3.125 to 25 µg/ml and 3.125 to 50 µg/ml respectively with varying effectiveness. TQ, TL, EU and AA showed significant inhibition up to 86.22%, 86.69%, 89.18 and 90.72% at the concentrations of 100, 87.5, 21.875 and 50 µg/ml respectively. Concentration for 50% inhibition of hemolysis (IC_{50}) of TQ, TL, EU and AA was 40, 32, 14 and 7.5 µg/ml respectively. Results were considered statistically significant at $p<0.005$. Inhibition of hemolysis was also observed at lower concentrations.

As reflected in Fig. 21, the onset of AAPH-induced hemolysis was significantly delayed in the presence of TQ, TL, EU and AA. Erythrocytes incubated with PBS at 37°C served as control and were stable with little hemolysis observed within 4 hour (4.61 ± 2.06%). When AAPH was added to the suspension of erythrocytes, hemolysis induction was time-dependent untill 3 hour and cause 96.5% hemolysis. The hemolysis is lagged at initial 2 hours. Addition of TQ, TL, EU and AA significantly protected the erythrocyte membrane from hemolysis induced by AAPH. Hemolysis caused by TQ, TL, EU and AA was found to be 93.1 ± 2.59%, 82.3 ± 2.49%, 35.6 ± 2.4% and 30.3 ± 2.31% respectively.

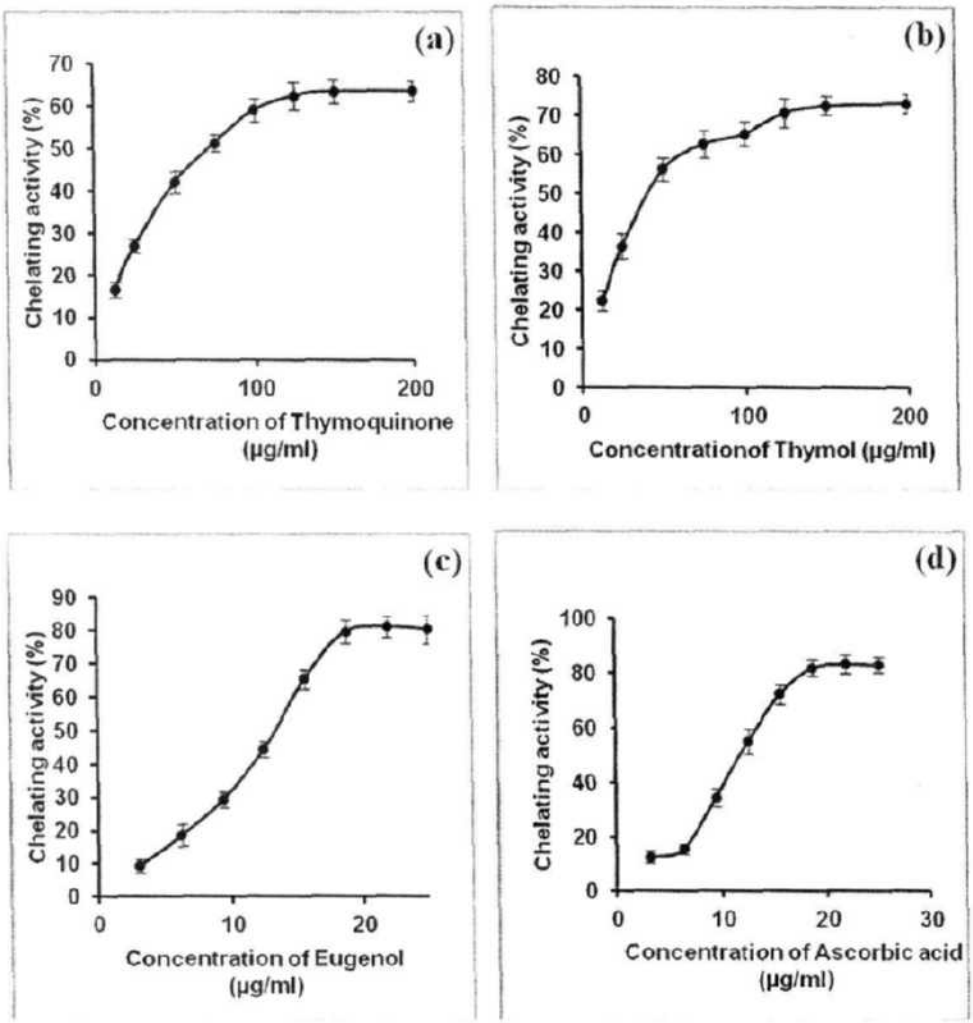

Fig. 19: Metal chelating activities of (a) TQ, (b) TL, (c) EU and (d) AA. All the data has been represented as Mean ± SD of triplicate samples.

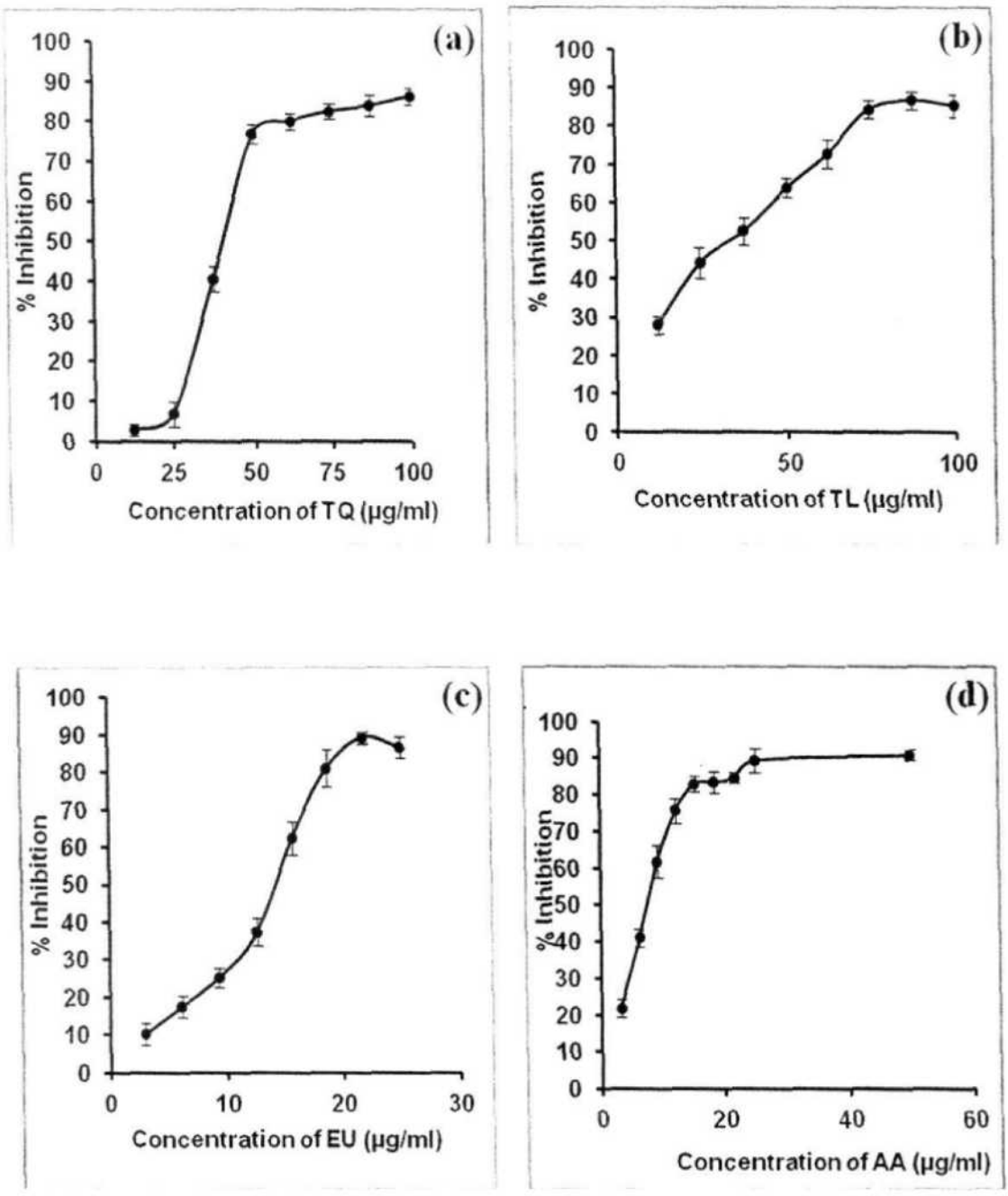

Fig. 20: Percentage inhibition of RBC lysis in AAPH assay by increasing concentrations of (a) TQ, (b) TL, (c) EU and (d) AA. All the data has been represented as Mean ± SD of triplicate samples.

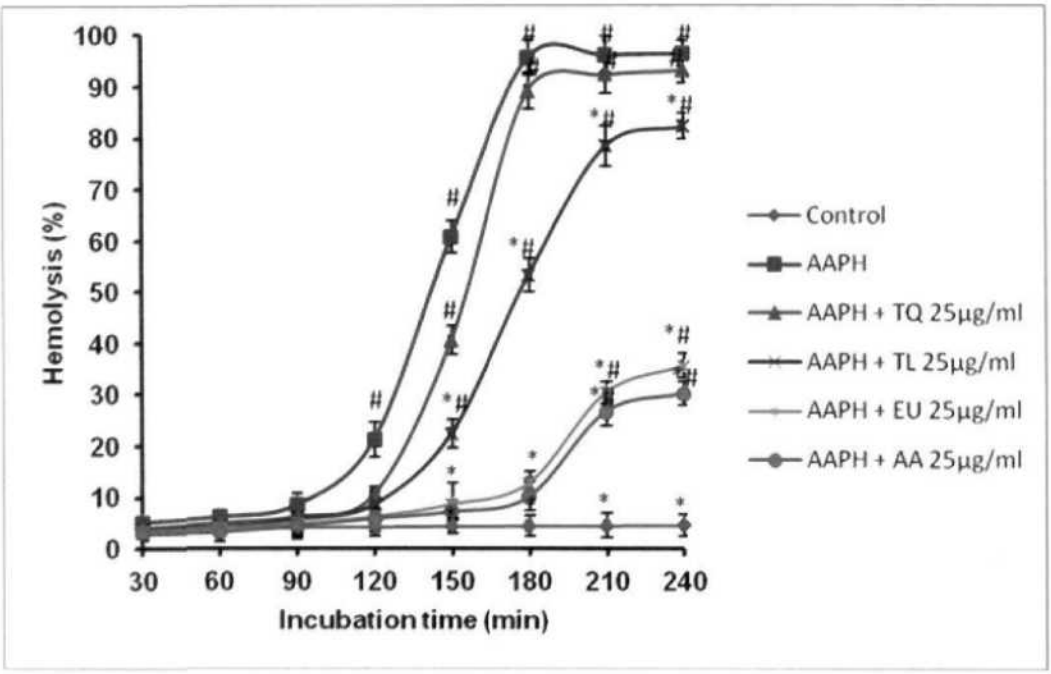

Fig. 21: Time dependent inhibition of AAPH-induced erythrocyte hemolysis by TQ, TL, EU and AA. 5% hematocrit was pre-incubated with TQ, TL, EU and AA at the 25 µg/ml of concentration for 30 min at 37°C. The cell suspension was then incubated with 200 mM AAPH for 4 hours at 37°C. In all experiments, control erythrocytes (without any treatment) and AAPH-treated erythrocytes (with AAPH) were used. All the data has been represented as Mean ± SD of three independent experiments.

*$p<0.05$, as compared with AAPH at respective time

#$p<0.05$, as compared with control at respective time

HSA, the most abundant protein in plasma, was selected as a model protein (Shaklai *et al.*, 1984). Glycation studies on HSA mainly focused on the reaction with glucose although some information with other sugars is also available. In the present study, the effect of glucose on HSA has been investigated for extended time period incubation. It is well recognized that the reaction of reducing sugars with protein can induce marked alterations in protein conformation. Absorption spectra continue to be used for the detection of sugar induced modification of protein (Yamauchi *et al.*, 2002). The glycation induced alterations in HSA were further examined with respect to their chromophoric and fluorophoric properties.

UV absorption spectral studies

The UV absorption spectra of glycated HSA incubated for 7 and 14 days showed hyperchromicity at 280 nm. The hyperchromicity at 7 and 14 days was 27.6% and 57.3% respectively, whereas glycated HSA incubated for 21 and 28 days showed hypochromicity at 280 nm to the extent of 30.8% and 34.9%. However, no apparent peak shift was found in all the cases (Fig. 22).

Fluorescence spectroscopy

The native and glycated HSA was characterized for its fluorescence emission spectra. Due to high sensitivity and reproducibility, the level of fluorescence of a single tryptophan residue in the HSA molecule has been chosen as an index of conformational changes. Tryptophan specific fluorescence analysis were conducted, where both native as well as glycated HSA samples were excited at 285 nm and emission spectra was taken over a range of 290-400 nm. As shown in Fig. 23, the emission maxima of native and glycated HSA samples were found to be 335 and 330 nm respectively indicating a blue shift of 5 nm for glycated HSA. The glycated HSA incubated for 7 and 14 days showed an increase in the magnitude of fluorescence intensity and the percent gain in fluorescence was 8.24% and 18.63%, whereas glycated HSA incubated for 21 and 28 days showed reduction in the fluorescence intensity and the loss in fluorescence intensity was 48.49% and 55.81%.

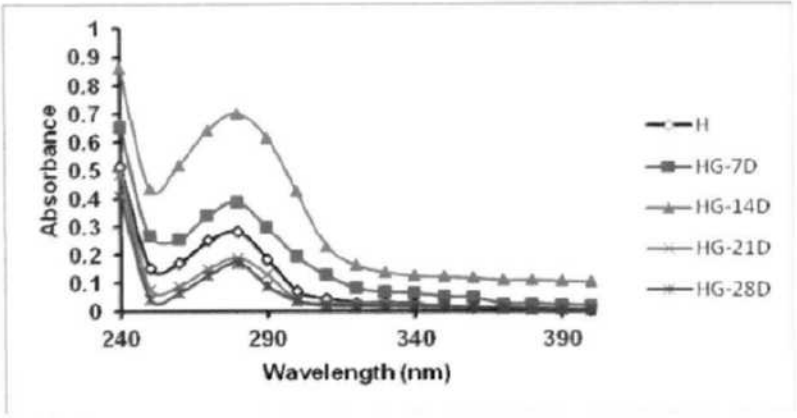

Fig. 22: Ultraviolet absorption spectra of native HSA (H) and glycated HSA (HG) at 7, 14, 21 and 28 days.

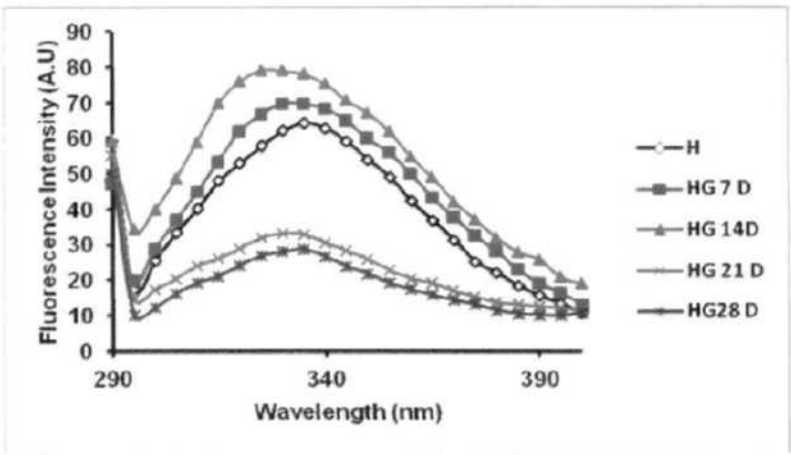

Fig. 23: Tryptophan fluorescence spectra of native HSA (H) and glycated HSA (HG) at 7, 14, 21 and 28 days.

AGE specific fluorescence

AGE specific fluorescence is widely used for the detection of AGEs and for following the onset of AGE formation both *in vitro* and *in vivo* (Nagai *et al.*, 2000; Valencia *et al.*, 2004). The fluorescence spectra recorded at an excitation wavelength of 370 nm is shown in Fig. 24. The glycated HSA incubated for 7 days did not show AGEs specific fluorescence at 370 nm (λex). Incubation of HSA with glucose for 14 and 21 days showed an increase in fluorescence as compared to control. Incubation beyond 21 days however, resulted in no further increase in fluorescence.

Circular dichroism (CD) spectroscopy

The Far-UV CD spectroscopy can also be used to study the secondary structure of proteins and their conformational changes. The Far-UV CD spectrum of HSA exhibited two negative bands in the ultraviolet region at 208 and 222 nm which is typical for alpha-helical structure (Fig. 25). The CD spectra obtained for native HSA and glycated HSA showed significant differences, indicating major changes in the secondary structure of glycated HSA at 7, 14, 21 and 28 days compared to native HSA. Incubation of HSA with glucose leads to a decline in the negative CD signal on 7 days at 208 nm and 222 nm as compared to control (Fig. 2). This negative CD signal started decreasing slightly with time in HSA samples incubated with glucose. Glycated HSA at 21 days showed further decrease in CD signal as compared to glycated HSA samples incubated for 14 days. Further incubation with glucose up to 28 days showed a gradual disappearance of the minima at 208 and 222 nm and appearance of minima between 210 and 220 nm occur. The α-helical content was 64% in native HSA. However, incubation of HSA with glucose showed decrease in α-helix content with time as shown in Table 4. The α-helical content of the protein decreased from 64% to 61%, 56%, 51% and 48% on 7, 14, 21 and 28 days of incubation of HSA with glucose.

FTIR spectroscopy

Finally, the AGEs were characterized by FTIR spectroscopy. FTIR spectra recorded for native (Fig. 26a) and glycated HSA (Fig. 26b) were analyzed on the basis of shape and frequency of the amide I and II bands and other characteristic group present in their structures. Spectra recorded the amide I peak at 1650.87 cm^{-1} which was consistent with the absorption peak of α-helix, amide II at 1571.51 cm^{-1} and epoxy ring at 813.6 cm^{-1} in HSA. Spectra recorded for glycated HSA show shift in amide I and amide II band and were observed at 1646.9 cm^{-1} and 1535.8 cm^{-1} respectively as compared with native HSA peaks. The interaction of HSA with glucose is attributed by peak observed at 1158.83 cm^{-1} which is ascribed to the asymmetrical stretching vibration of C-O-C formed by the epoxy ring opening.

Table 4: CD Characteristics of native and glycated HSA

Groups		*MRE at 222 nm	Alpha helix
Native HSA		-17125.3	64%
Glycated HSA	7 days	-16061.5	61%
	14 days	-14824.1	56%
	21 days	-13242.6	51%
	28 days	-12184.5	48%

*MRE measured in deg cm^2 mol^{-1}

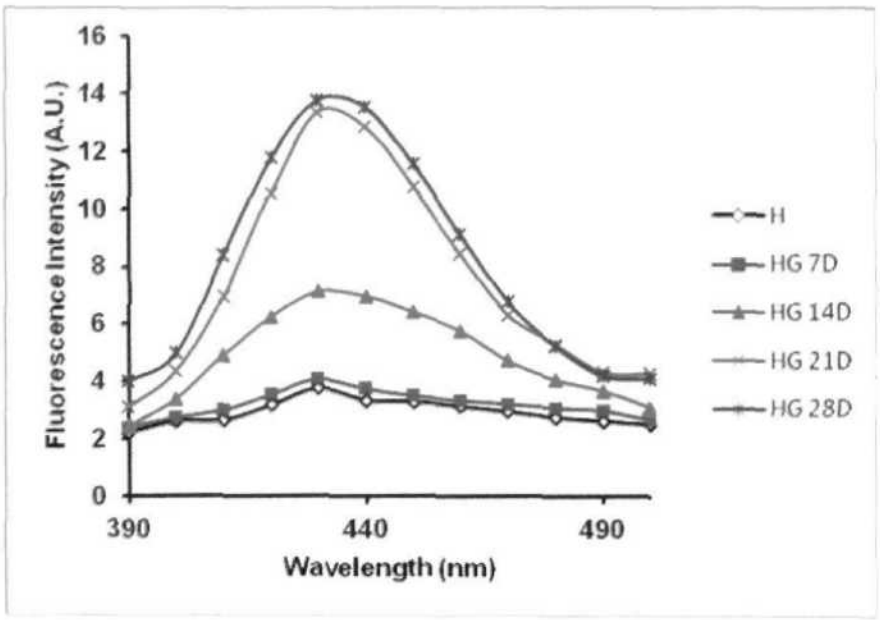

Fig. 24: AGE specific fluorescence spectra of native HSA (H) and glycated HSA (HG) at 7, 14, 21 and 28 days.

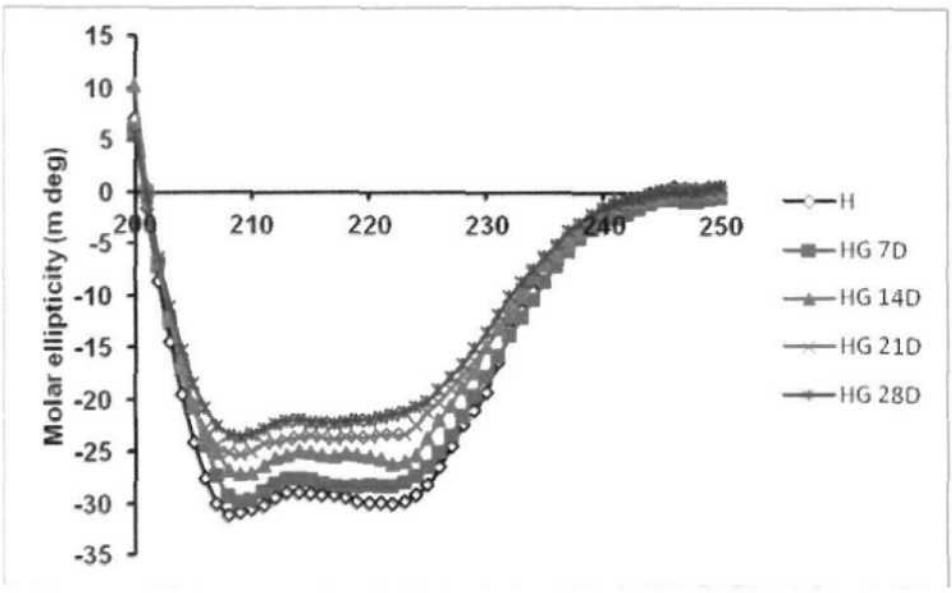

Fig. 25: Circular dichroism spectra of native HSA (H) and glycated HSA (HG) at 7, 14, 21 and 28 days.

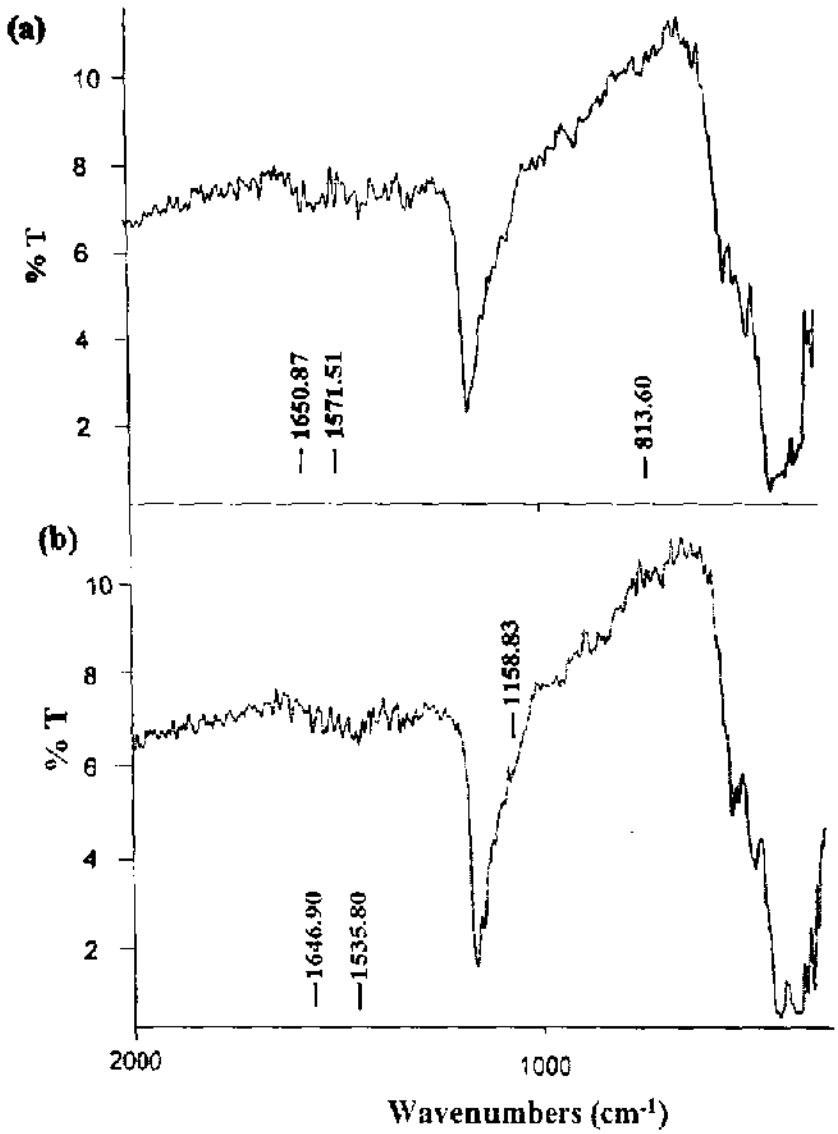

Fig. 26: FTIR Spectra of (a) native HSA (b) HSA glycated with 100 mM glucose.

Ketoamine estimation

Ketoamines are early non-enzymatic glycation adducts and are important precursors of AGEs and hydroxyl radicals (Ahmed., 2002). The ketoamine moieties formed by the glycation of HSA incubation for 7 days were measured colorimetrically by using NBT. The formation of ketoamine was found to be 61.12 ± 2.68 nmoles/mg of protein for glycated HSA. The non-glycated HSA gave a negligible ketoamine concentration of 3.14 ± 0.26 nmoles/mg of protein (Fig. 27).

Protein bound carbonyl groups

Carbonyl content is considered a reliable indicator of glycation and is the most commonly used marker of protein oxidation and glycation. Modified HSA was analyzed for carbonyl content by its reaction with DNPH, for assessing glycoxidative modification. Significant amounts of carbonyl groups were produced on glycation. The carbonyl contents of glycated HSA was detected to be 3.6 ± 0.3, 5.9 ± 0.13, 8.06 ± 0.22 and 8.07 ± 0.26 nmoles/mg of protein at day 7, 14, 21 and 28 post incubation respectively. The carbonyl content went on increasing till day 21 and after that there was no further increase (Fig. 28). HSA incubated without sugar contained very small amount of carbonyl groups (1.73 ± 0.58 nmoles/mg of protein).

Determination of free amino groups

HSA incubated with glucose for 7-28 days was also studied for loss of free amino groups. Free amino group content in glycated HSA decreased significantly in response to incubation with glucose. Percent decrease in free amino groups of glycated HSA was found to be 7.65 ± 0.24, 9.87 ± 0.44, 15.51 ± 0.50 and 15.91 ± 0.50 at 7, 14, 21 and 28 days of incubation as compared to native HSA (Fig. 29). It clearly shows that as the time increases there was a decrease in free amino group of glycated HSA up to 21 days. The loss of free amino groups of glycated HSA was same on day 21 and 28 of incubation.

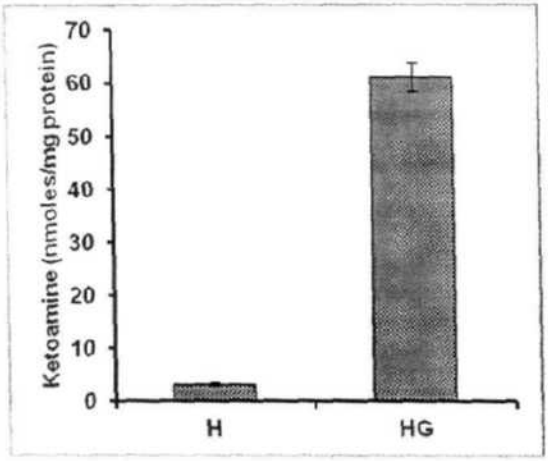

Fig. 27: Level of ketoamines in native HSA (H) and glycated HSA (HG) after 7 days of incubation time. All the data has been represented as Mean ± SD for samples in triplicate.

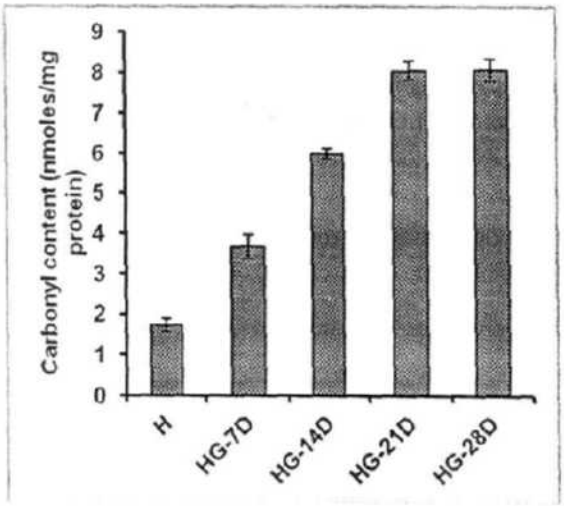

Fig. 28: Carbonylation of native HSA (H) and glycated HSA (HG) at 7, 14, 21 and 28 days of incubation. All the data has been represented as Mean ± SD of triplicated samples.

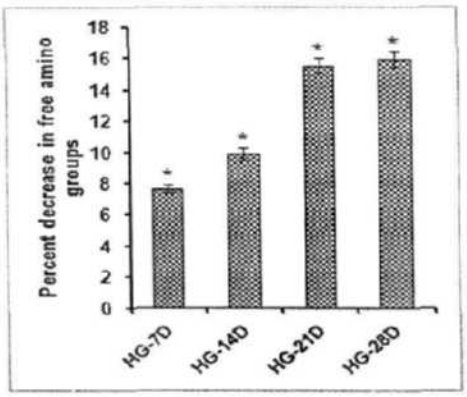

Fig. 29: Percent decrease in amino groups of HSA incubated with 100 mM glucose for 7, 14, 21 and 28 days. All the data has been represented as Mean ± SD of triplicated samples. *Significantly different at $p<0.05$ from native HSA.

Gel Electrophoresis

Native and glycated HSA was analyzed by 10% SDS-PAGE in the presence of the thiol reductant β-mercaptoethanol. Fig. 30(a), demonstrates the SDS-PAGE analysis of HSA incubated with glucose from 7 days to 28 days. HSA not exposed to glucose migrated as a single band of about 66 kD but on incubation with glucose, clear alteration in the electrophoretic behavior was evident. These included broadening of the protein band towards high and low molecular weight suggesting the formation of high and low molecular weight aggregates. The second notable effect was decrease in the staining intensity of the original bands. These sugar-induced alterations were more obvious at 21 and 28 days of glycation.

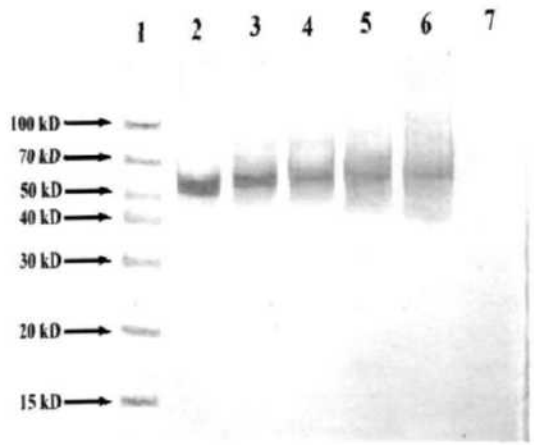

Fig. 30 (a): SDS-PAGE of HSA incubated with glucose (100 mM) in 20 mM phosphate buffer, pH 7.4 at 37°C for 7, 14, 21 and 28 days.

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose incubated for 7 days

Lane 4: HSA + Glucose incubated for 14 days

Lane 5: HSA + Glucose incubated for 21 days

Lane 6: HSA + Glucose incubated for 28 days

Densitometric analysis of SDS-PAGE gels

The area selected for densitometric analysis of glycated HSA and native HSA was same, so that comparison can be easily done. A portion of band selected for native HSA showed relative density of 608.6, while the equal portion of band selected for glycated HSA at 7, 14, 21 and 28 days of incubation showed 598.2, 590.3, 523.2 and 512.6 relative density respectively (Fig. 30b). Density of bands incubated with glucose was much less than that of native HSA.

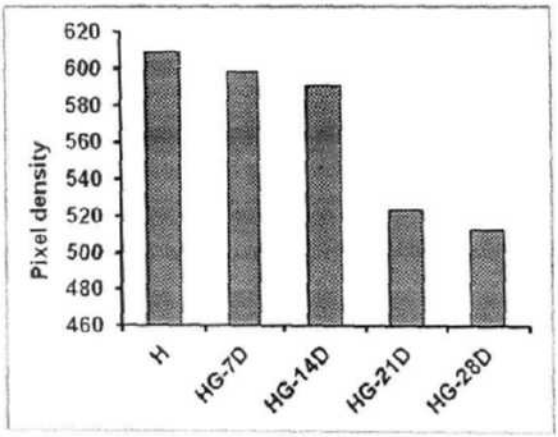

Fig. 30 (b): Densitometric analysis of SDS-PAGE. HSA was incubated in absence and presence of glucose (100 mM) in 20 mM phosphate buffer, pH 7.4 at 37°C for 7, 14, 21 and 28 days.

The aim for this investigation was to compare the inhibitory effects of various concentrations of phytochemicals viz. thymoquinone (TQ), thymol (TL) and eugenol (EU) upon HSA glycation *in vitro*. This information was fetched by estimating level of ketoamines, free lysine residues, protein carbonyl content and studying protein fragmentation in glycated HSA treated with various concentrations of phytochemicals (TQ, TL and EU).

Studies on effect of phytochemicals on UV-Visible Spectrophotometry

AG (1 mM), TQ (3, 30 and 300 µM), TL (3, 30 and 300 µM) and EU (0.06, 0.6 and 6.0 µM) showed antiglycation action after various time periods when percent chromicity of the samples was calculated at 280 nm (Table 5). Glycated HSA showed hyperchromicity of 27.6% and 57.3% after 7 and 14 days of incubation and 30.79% and 34.9% hypochromicity after 21 and 28 days of incubation respectively (Fig. 22). Hyperchromicity was found to be decreased for AG treated glycated HSA samples as compared to glycated HSA after 7 and 14 days. Similarly, reduction in hypochromicity was also observed to AG treated glycated HSA samples after 21 and 28 days of incubation in comparison to glycated HSA. EU-2 showed highest reduction in hyper and hypochromicity in comparison to other formulations of its group. However, TQ-2 and TL-2 exhibited better reduction in hyper and hypochromicity when compared to other preparations of their respective groups (Fig. 31-46). As compared to TQ-2 and TL-2, EU-2 showed better results (Fig. 47).

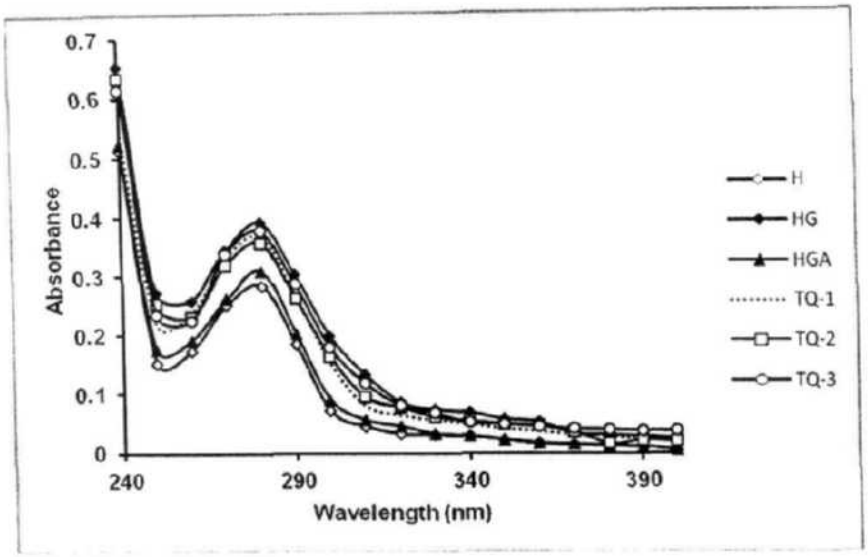

Fig. 31:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 7 days.

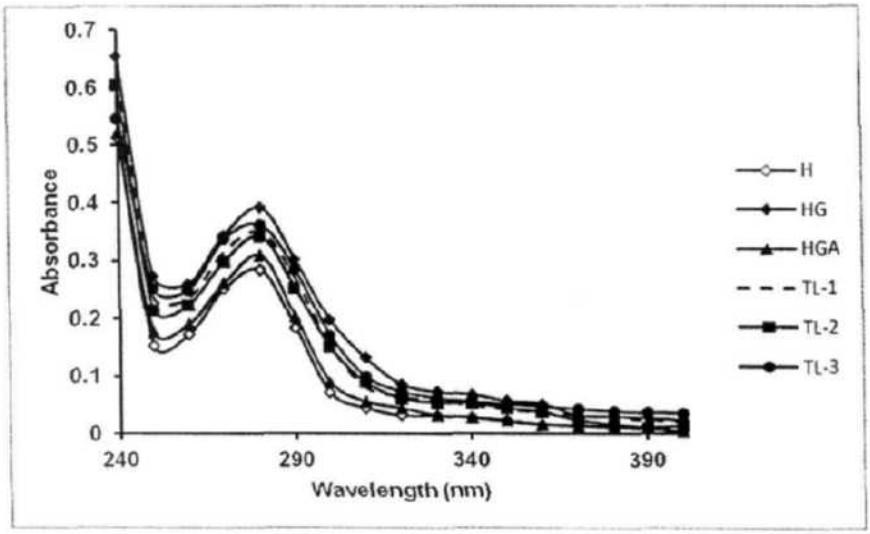

Fig. 32:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 7 days.

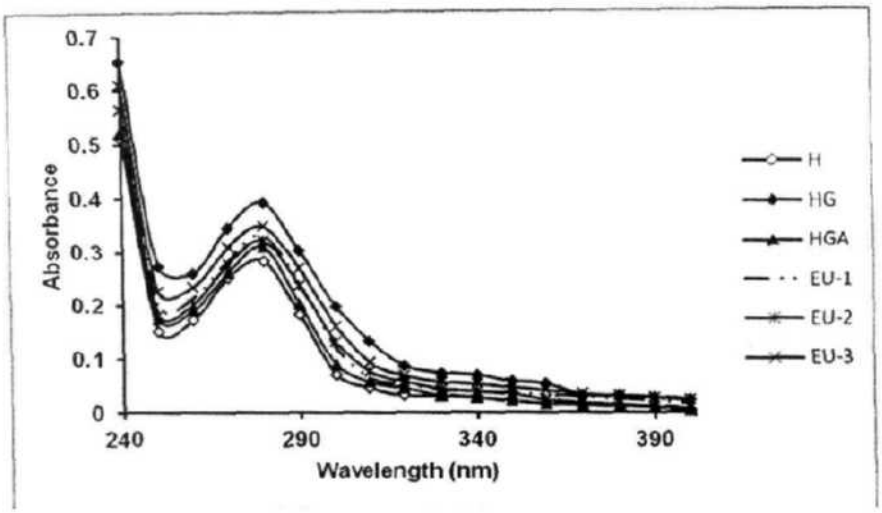

Fig. 33:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 7 days.

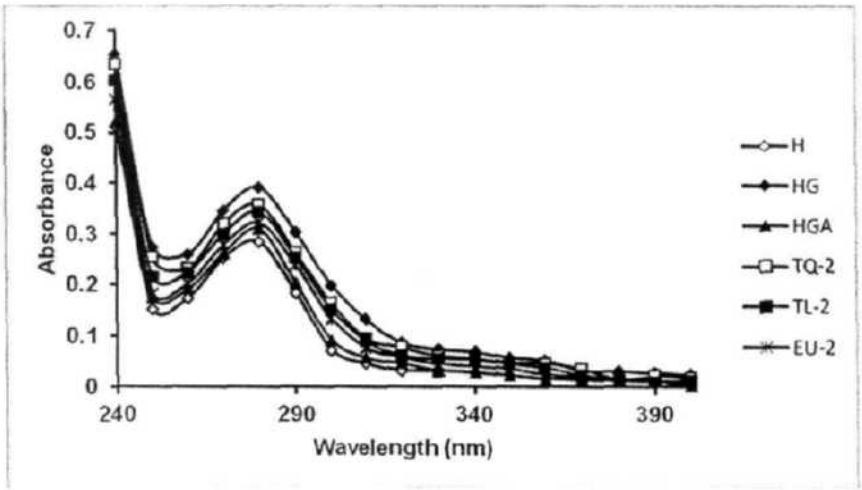

Fig. 34:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 7 days.

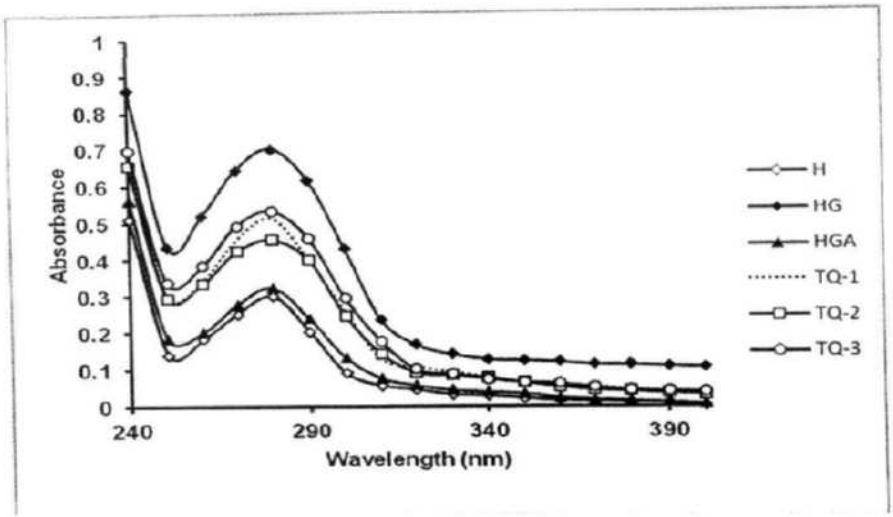

Fig. 35:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 14 days.

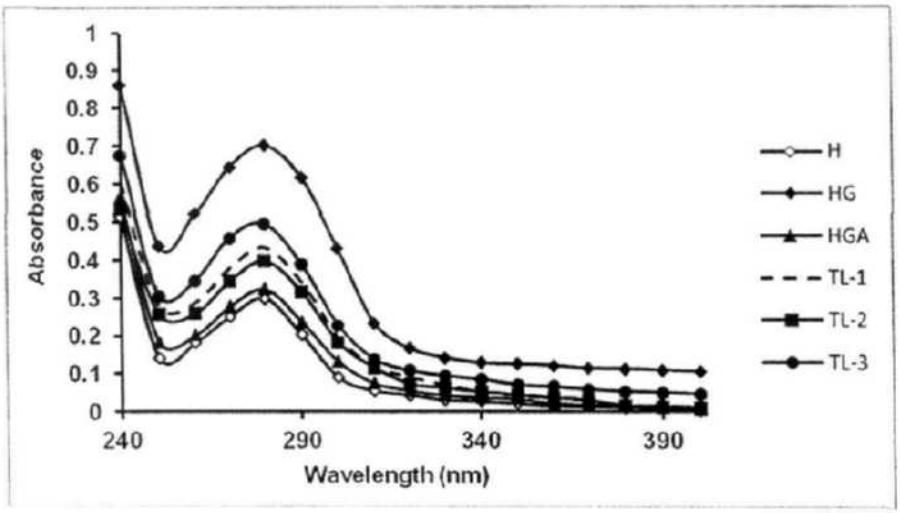

Fig. 36:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 14 days.

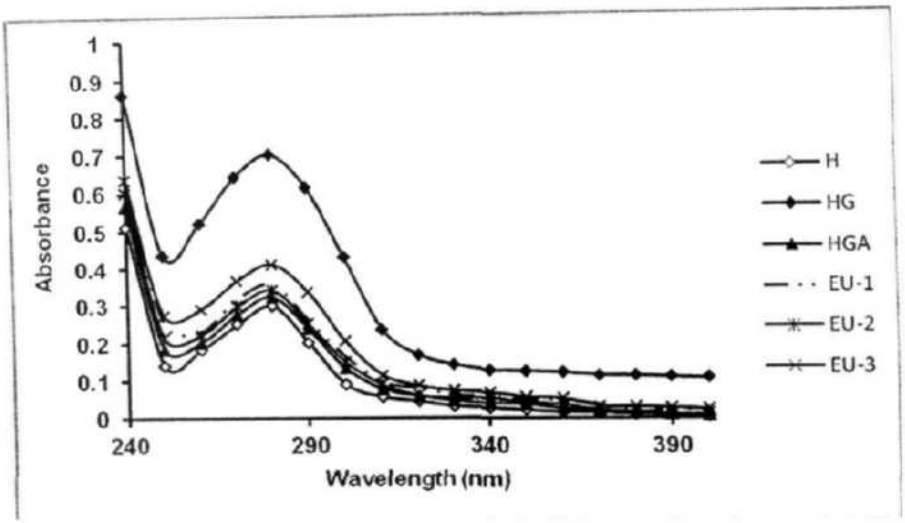

Fig. 37:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 14 days.

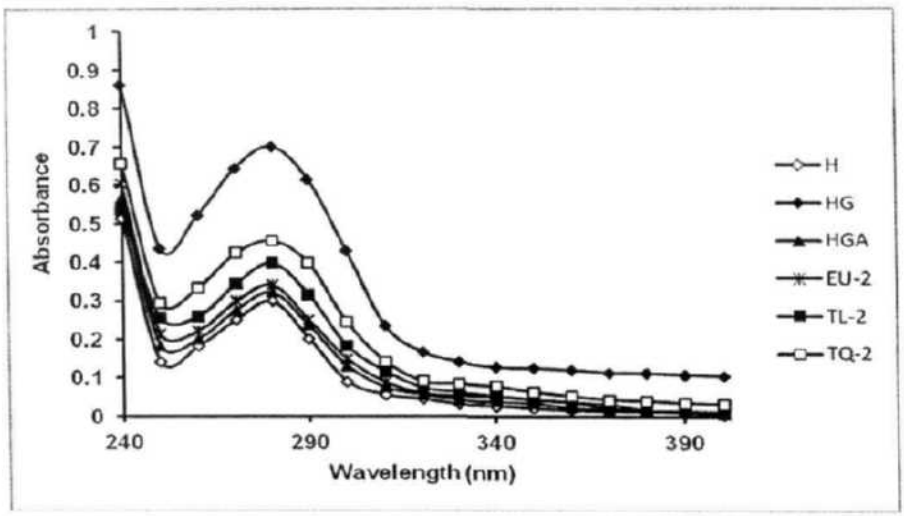

Fig. 38:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 14 days.

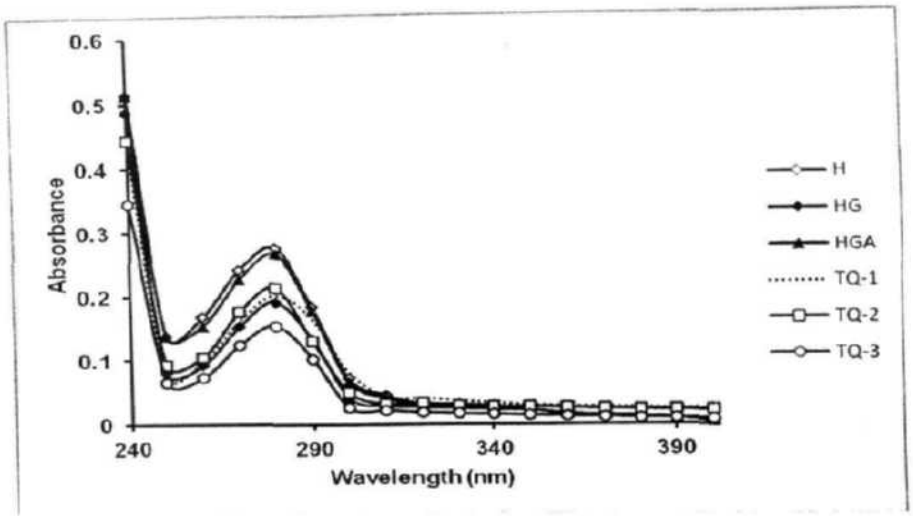

Fig. 39:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 21 days.

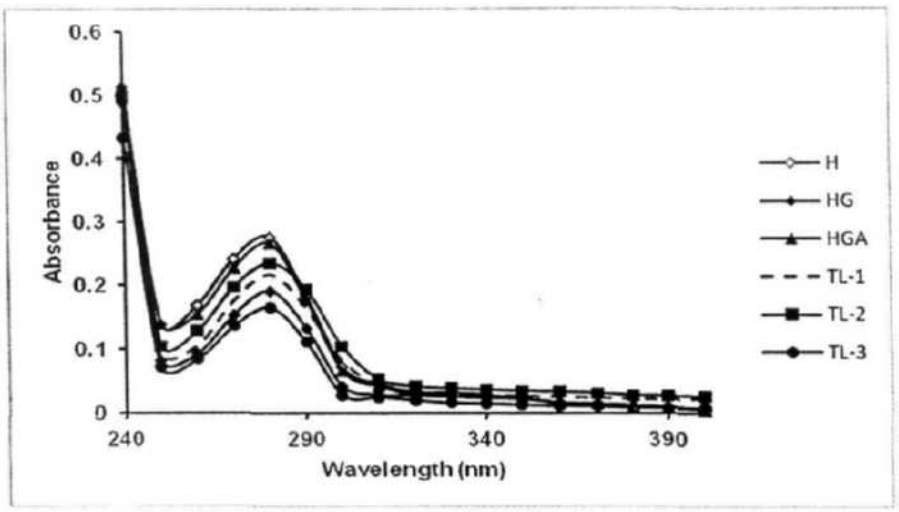

Fig. 40:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 21 days.

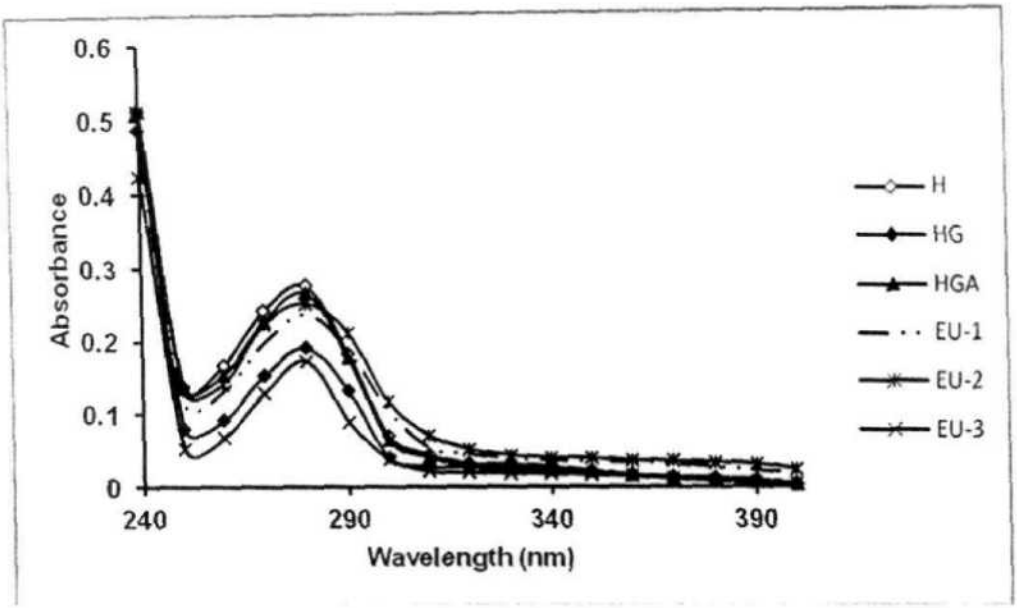

Fig. 41:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 21 days.

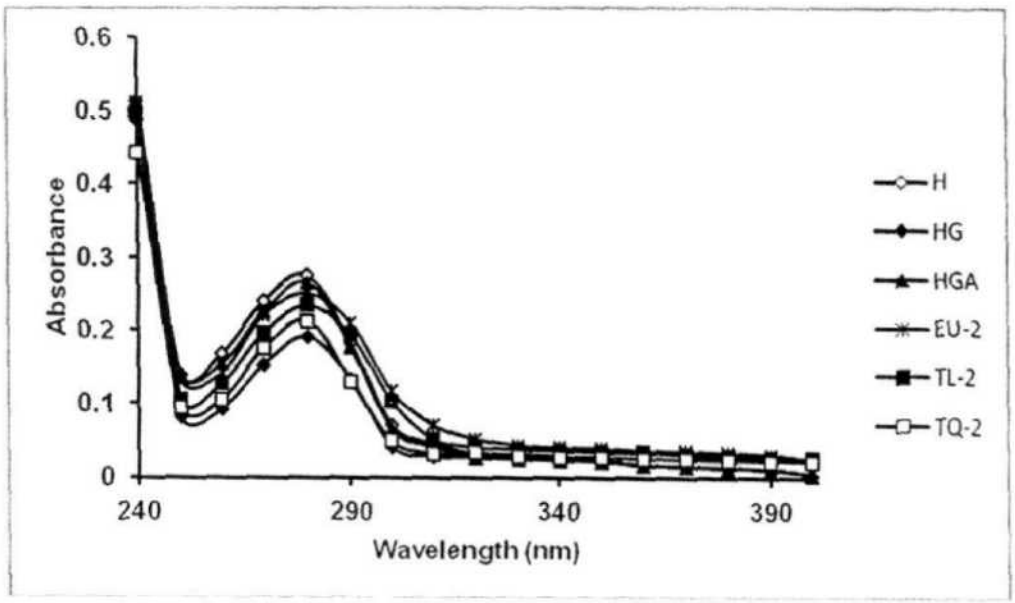

Fig. 42:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 21 days.

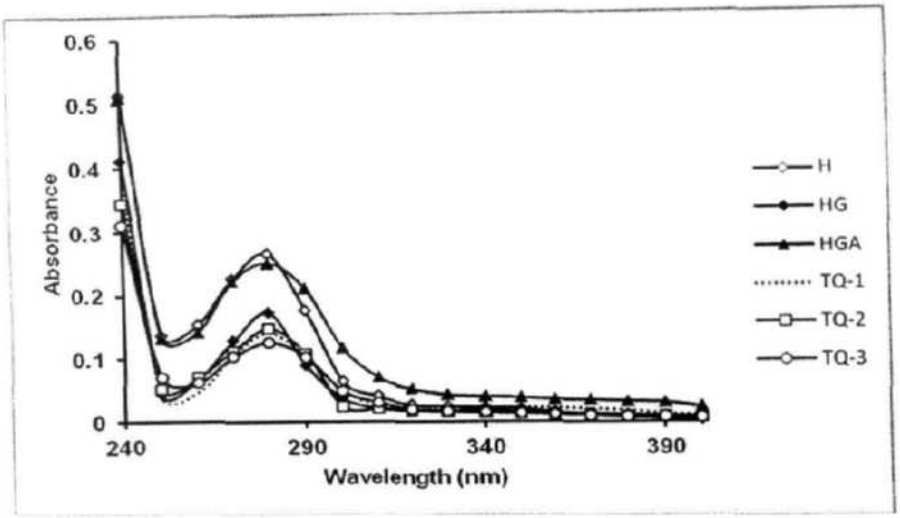

Fig. 43:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 28 days.

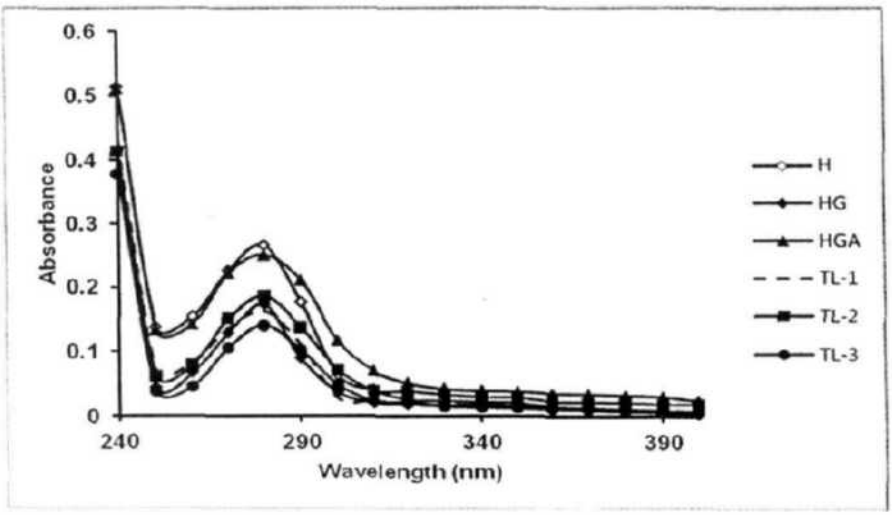

Fig. 44:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 28 days.

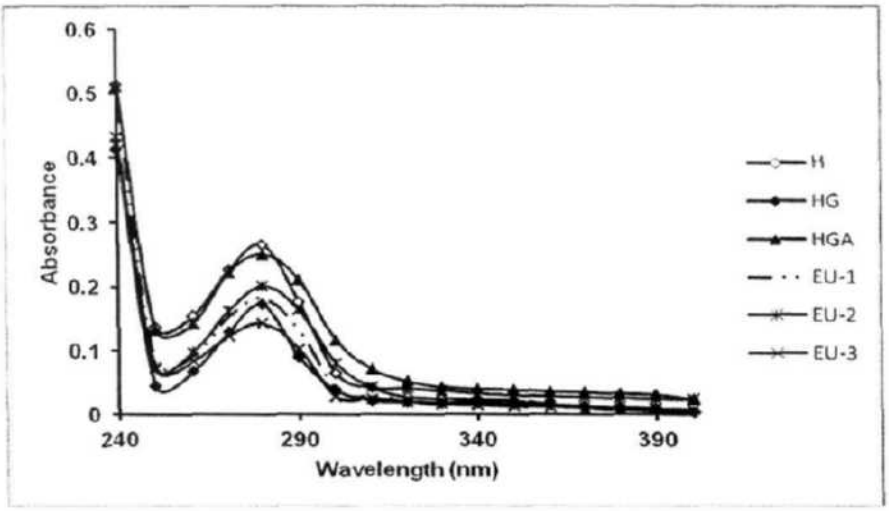

Fig. 45:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 28 days.

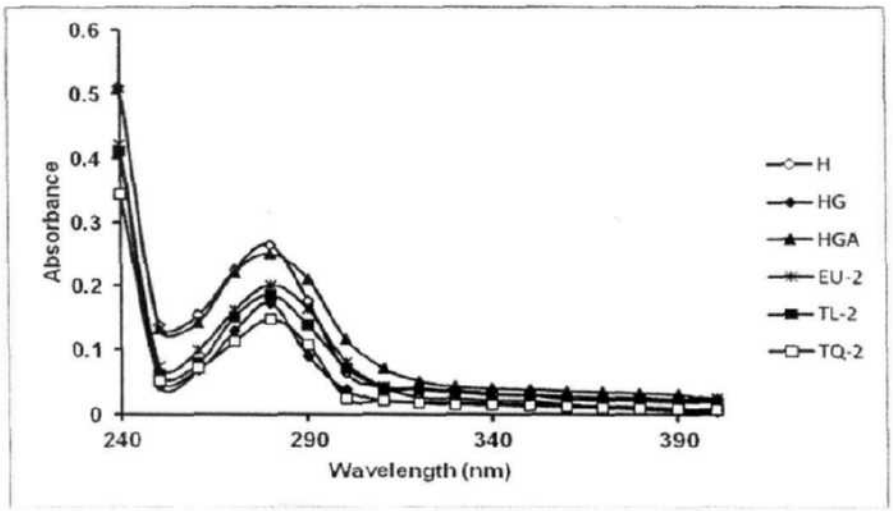

Fig. 46:- Ultraviolet absorption spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 28 days.

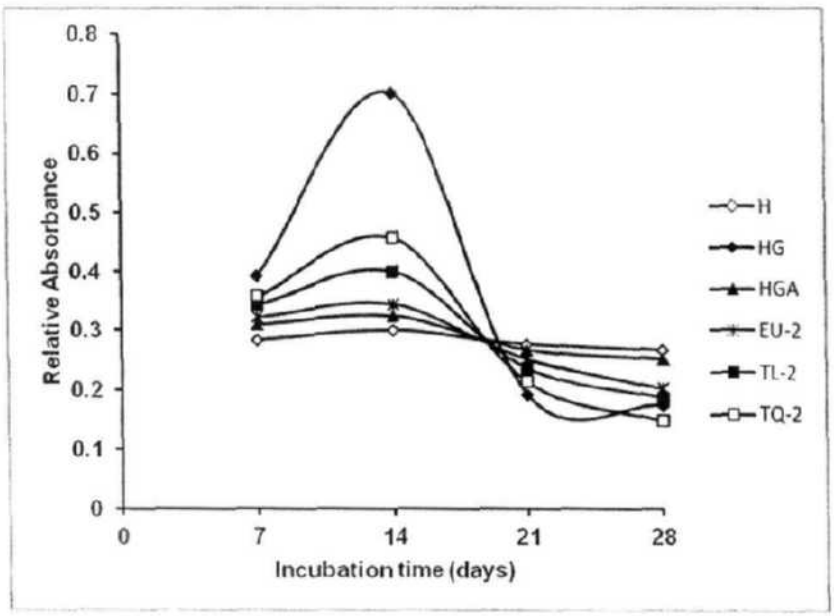

Fig. 47: Relative absorbance at 280 nm of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) on 7th, 14th, 21st and 28th day of incubation.

Table 5: UV Characteristics of HSA samples incubated with glucose.

Sample	Percent hyperchromicity at 280 nm		Percent hypochromicity at 280 nm	
	7 days	**14 days**	**21 days**	**28 days**
HG	27.62	57.35	30.79	34.96
HGA	8.41	7.43	3.62	5.64
TQ-1	23.1	41.83	26.81	46.99
TQ-2	20.73	34.43	22.83	44.36
TQ-3	24.93	43.9	44.2	52.63
TL-1	18.91	31.1	21.38	38.72
TL-2	17.25	24.87	14.85	30.07
TL-3	21.6	39.47	40.22	46.99
EU-1	14.5	16.25	14.49	31.2
EU-2	11.84	12.57	9.06	24.06
EU-3	18.68	27.25	37.32	46.24

Effect of various concentrations of phytochemicals on the percent gain and loss in the tryptophan fluorescence (intrinsic) intensity of glycated HSA

Percent gain and loss in the tryptophan fluorescence intensity (F.I.) of glycated HSA incubated with TQ, TL or EU after various time periods viz. 7, 14, 21 and 28 days was also analysed (Table 6). Fluorescence intensity was measured at 335 nm. Glycated HSA exhibited 8.24% and 18.63% gain in F.I. after 7 and 14 days and 48.49% and 55.81% loss in F.I. after 21 and 28 days respectively (Fig. 23). In concordance with UV results, both gain and loss in F.I. was observed to be decreased for AG treated HSA as compared to glycated HSA after each time point and the decrease seen in this case was highest amongst all the preparations tested. EU preparations followed AG in inducing reduction in gain as well as loss in F.I. EU-2 preparation exhibited highest decrease in gain and loss in F.I. in comparison to other formulations of its group. Similarly, TQ-2 and TL-2 showed better reduction in F.I. loss and gain when compared to other preparations of their respective groups (Fig. 48-63). EU-2 again was observed to be better than TQ-2 and TL-2 (Fig. 64).

Effect of varying concentration of TQ, TL and EU on the formation of AGEs from HSA glycation induced by glucose

Table 7 shows the effect of glucose on total AGEs formation in presence of AG, TQ, TL and EU at different concentration during 14, 21 and 28 days of HSA incubation. As it is evident from Table 7, TQ, TL and EU at different concentration has significantly decreased the formation of AGEs at all the time points. AG at a concentration of 1 mM was found to exhibit highest reduction in AGEs formation at all the time points as compared to other agents taken. TQ at a concentration of 30 µM showed a good decrease in protein carbonyl levels at each time point. Both 3 µM and 300 µM of TQ although could induce a reduction in AGEs formation but not as effectively as TQ-2. TL preparations were found to perform better in decreasing AGE-related fluorescence as compared to TQ preparation. The EU preparations at all three concentrations and at all the time points were found to be capable of suppressing AGEs formation better than the corresponding TQ and TL formulations (Fig. 65-76). In that respect, EU-2 (36.7%) is almost comparable to the effect of AG (42.08%). Parallel to the protein carbonyl results, EU-2 i.e. 0.6 µM of EU was found to reduce AGE fluorescence better than TQ-2 and TL-2 (30 µM of TQ and TL) after each time point (14, 21 and 28 days) (Fig. 77).

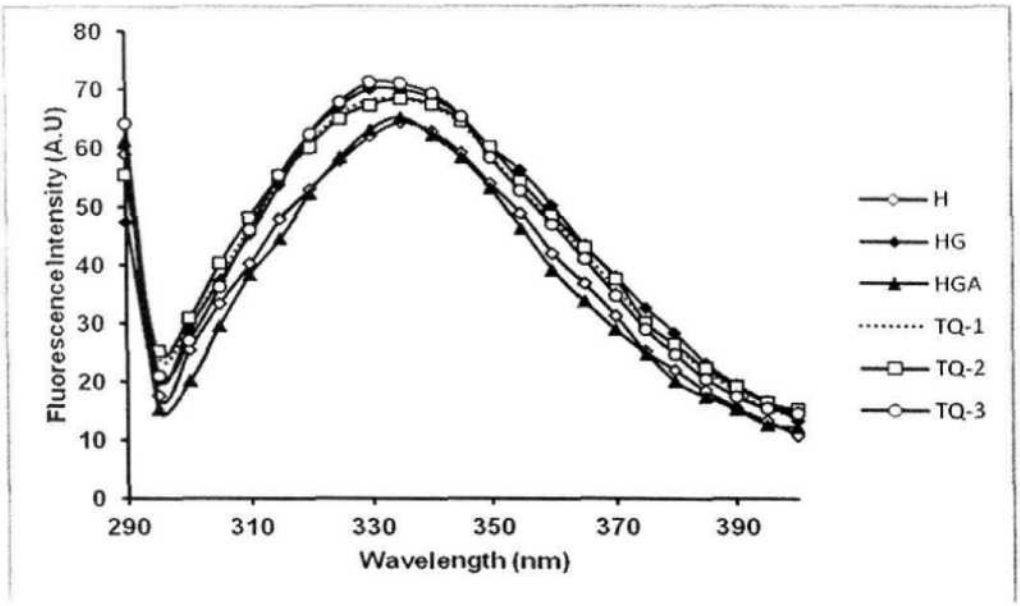

Fig. 48:- Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 7 days.

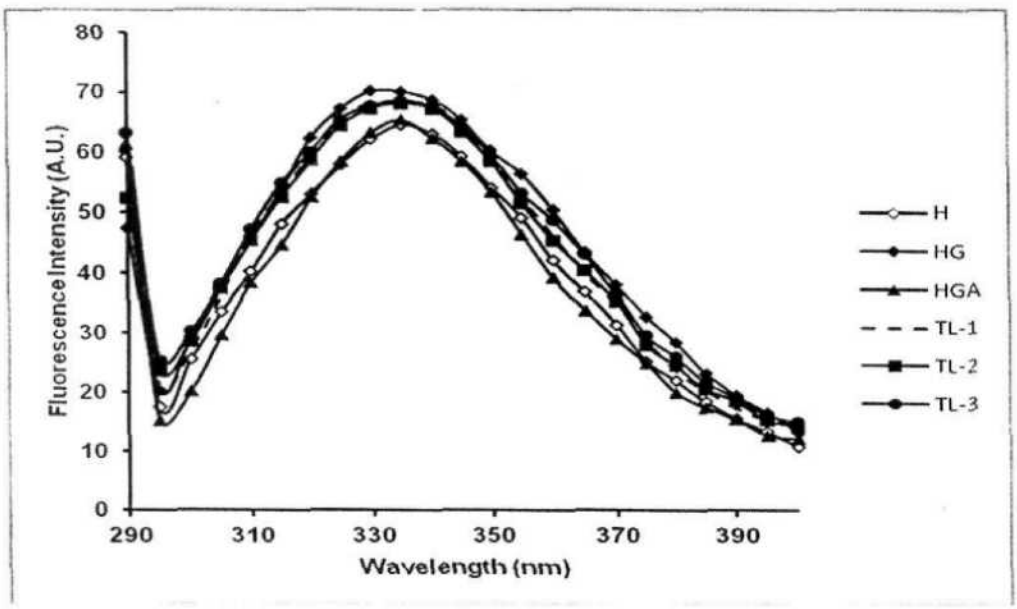

Fig. 49: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 7 days.

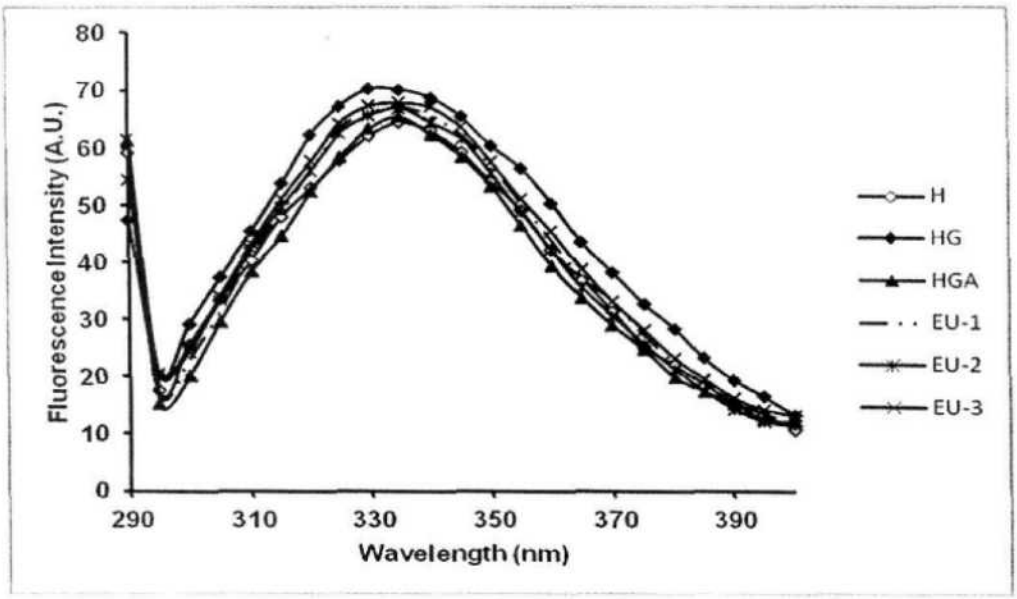

Fig. 50: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 7 days.

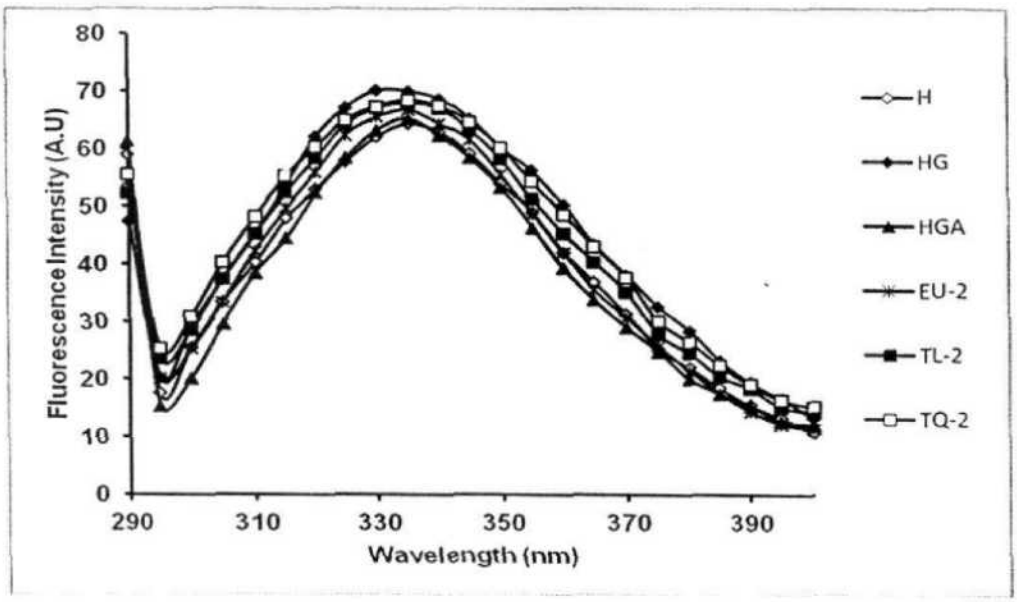

Fig. 51: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 7 days.

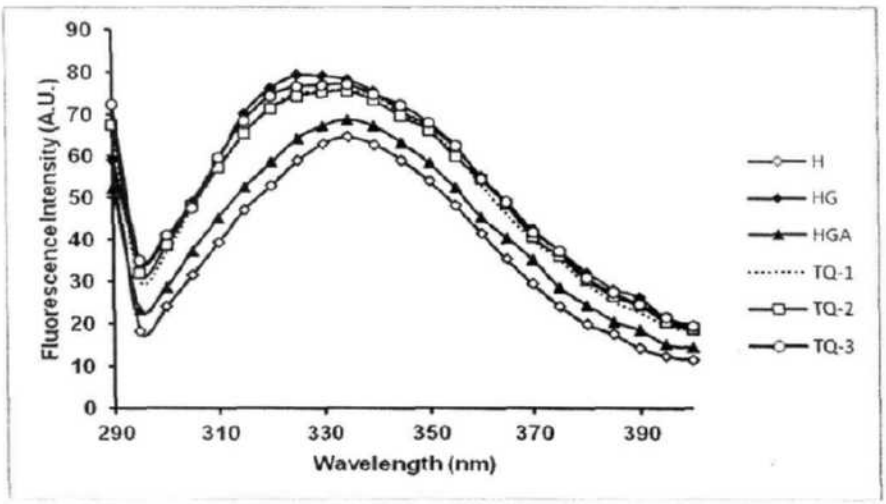

Fig. 52: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 14 days.

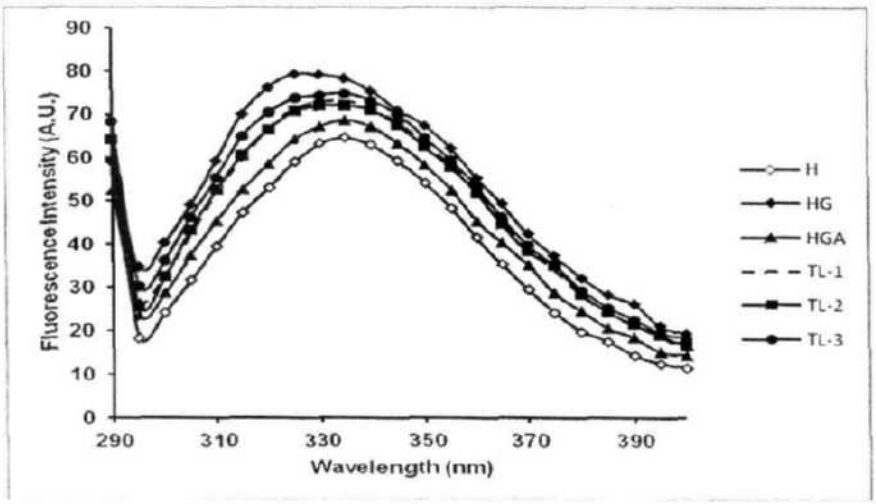

Fig. 53: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 14 days.

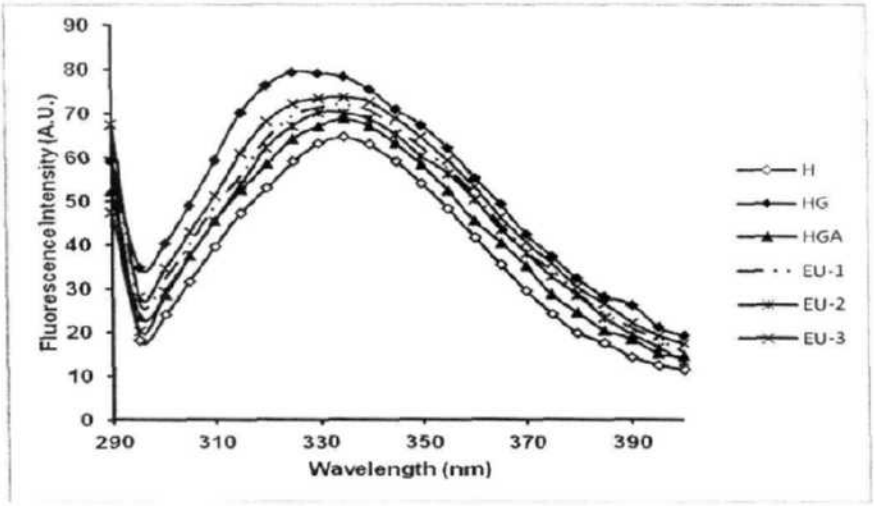

Fig. 54: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 14 days.

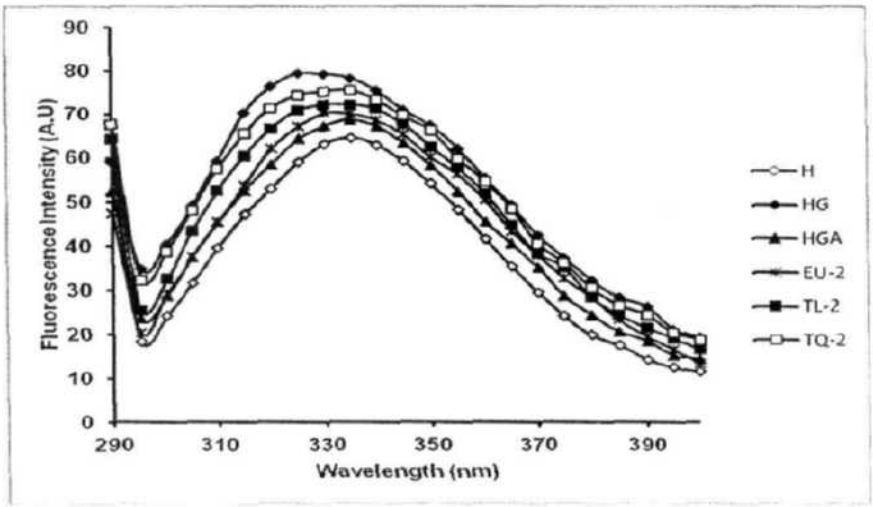

Fig. 55: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 14 days.

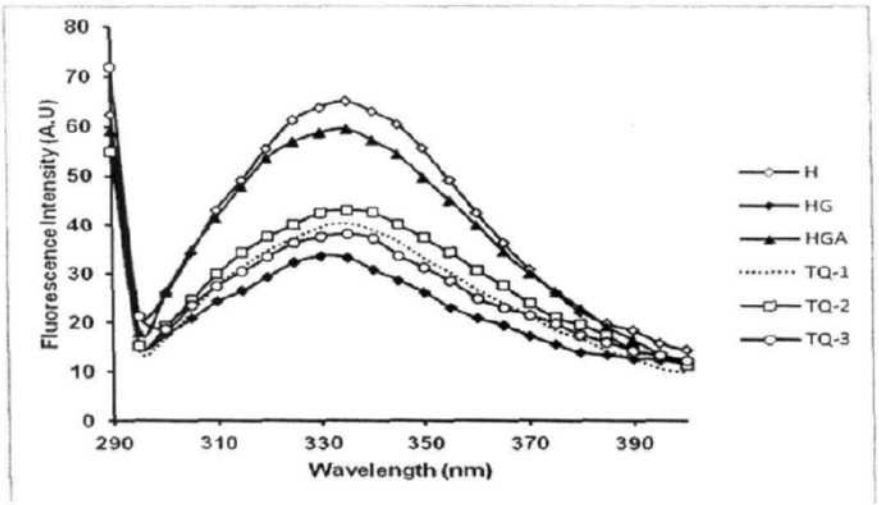

Fig. 56: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 21 days.

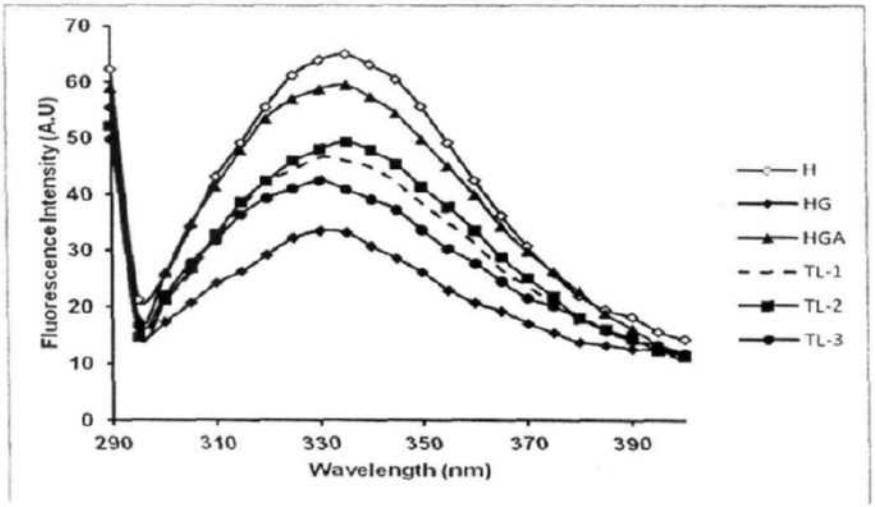

Fig. 57: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 21 days.

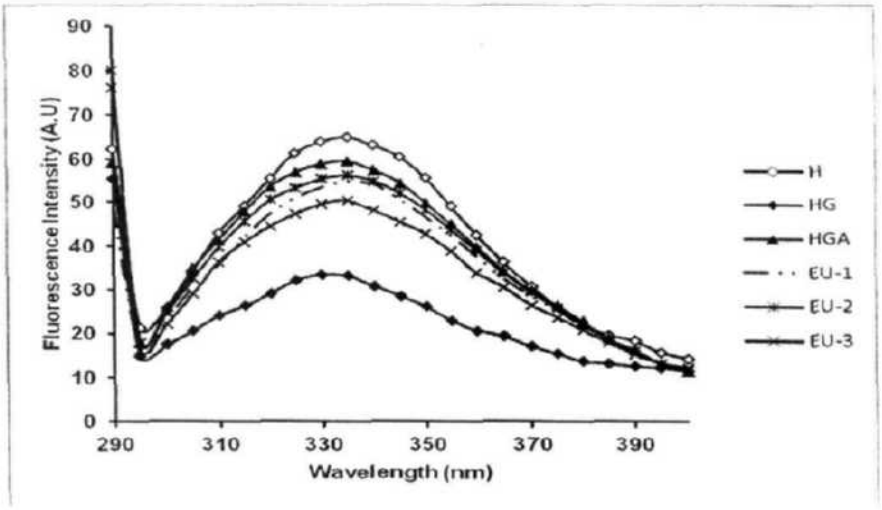

Fig. 58: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 21 days.

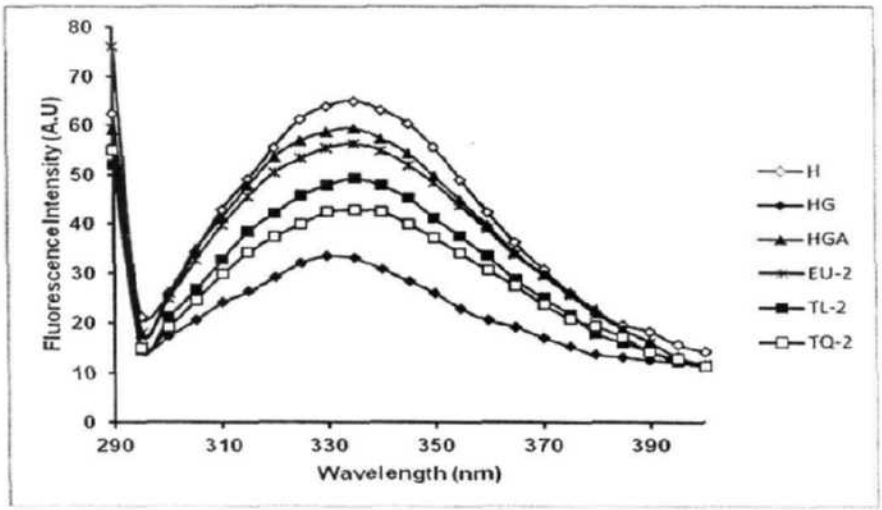

Fig. 59: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 21 days.

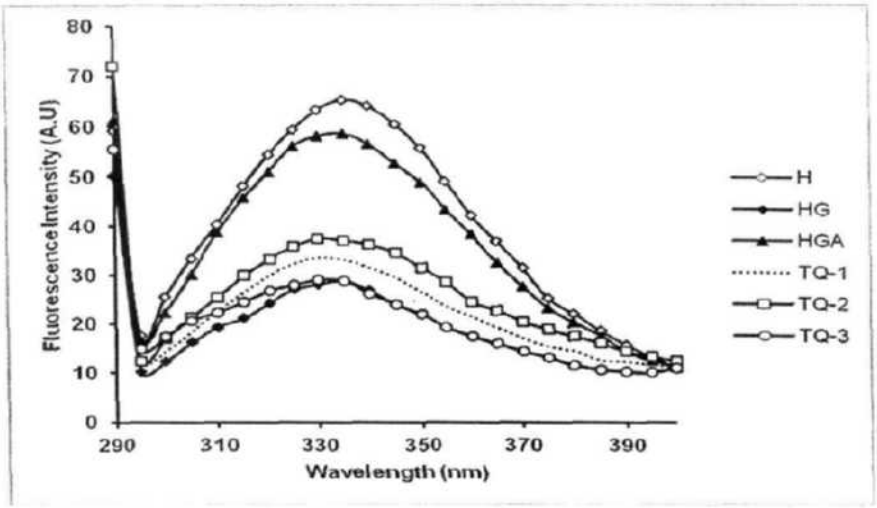

Fig. 60: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 28 days.

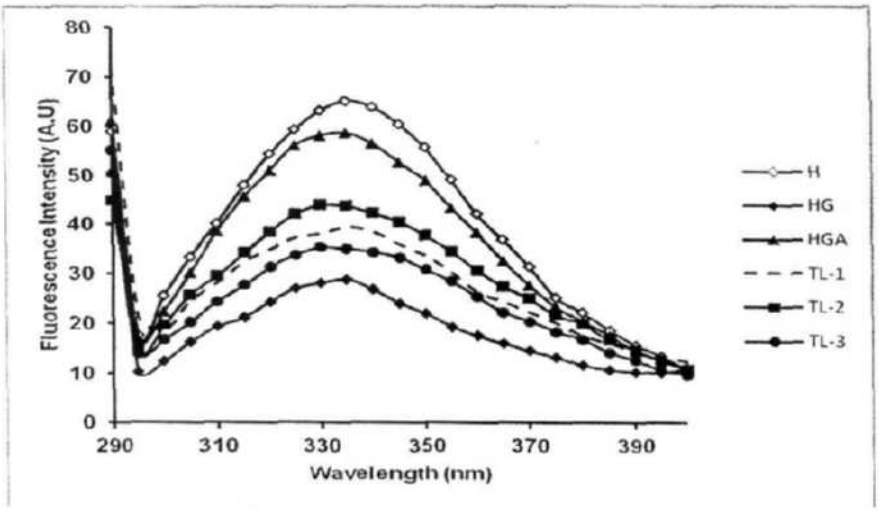

Fig. 61: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 28 days.

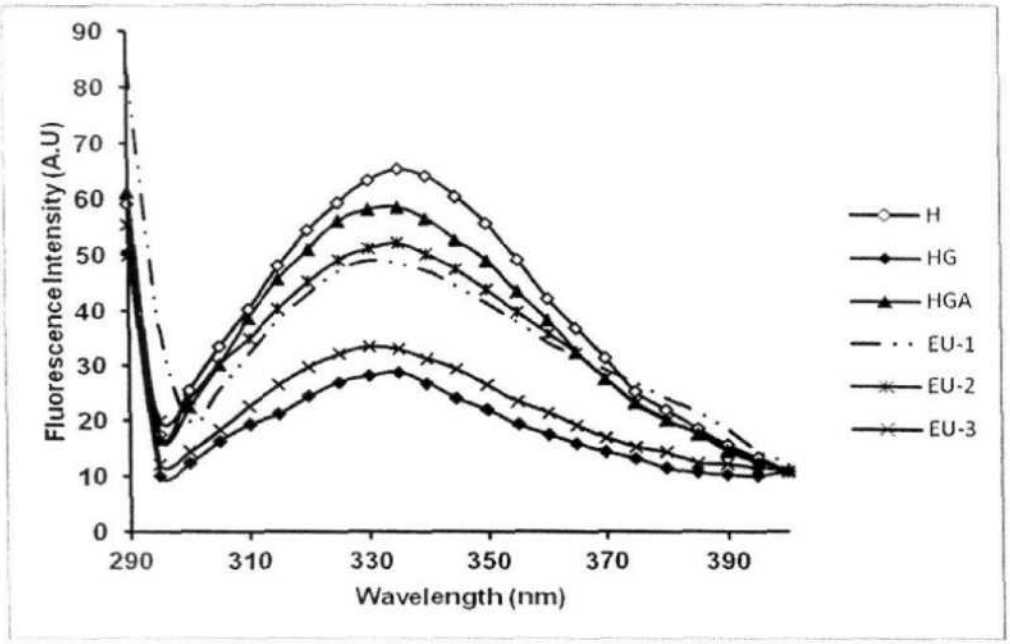

Fig. 62: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 28 days.

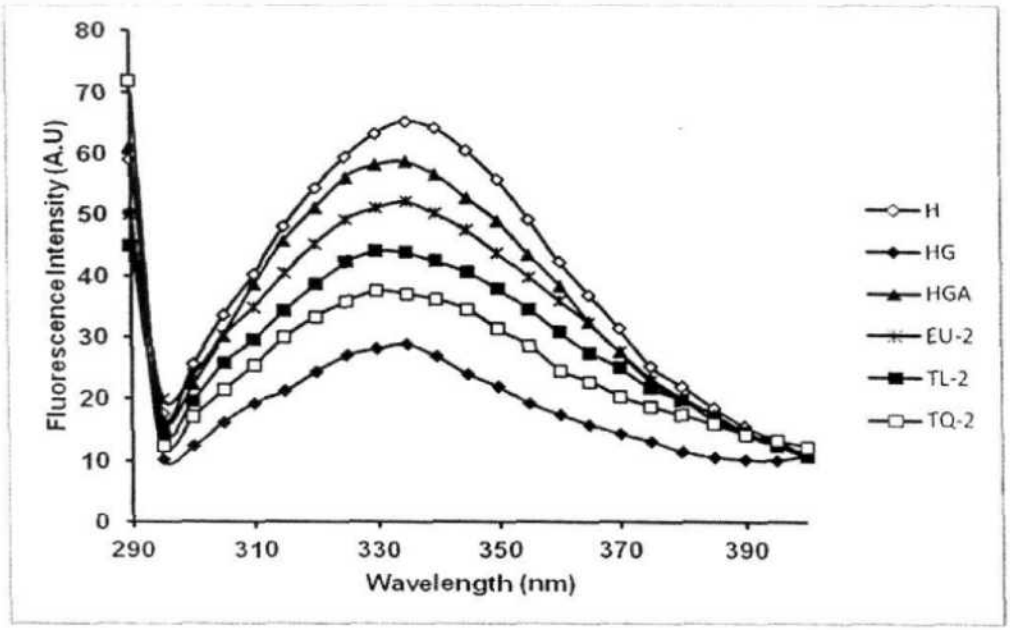

Fig. 63: Tryptophan fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 28 days.

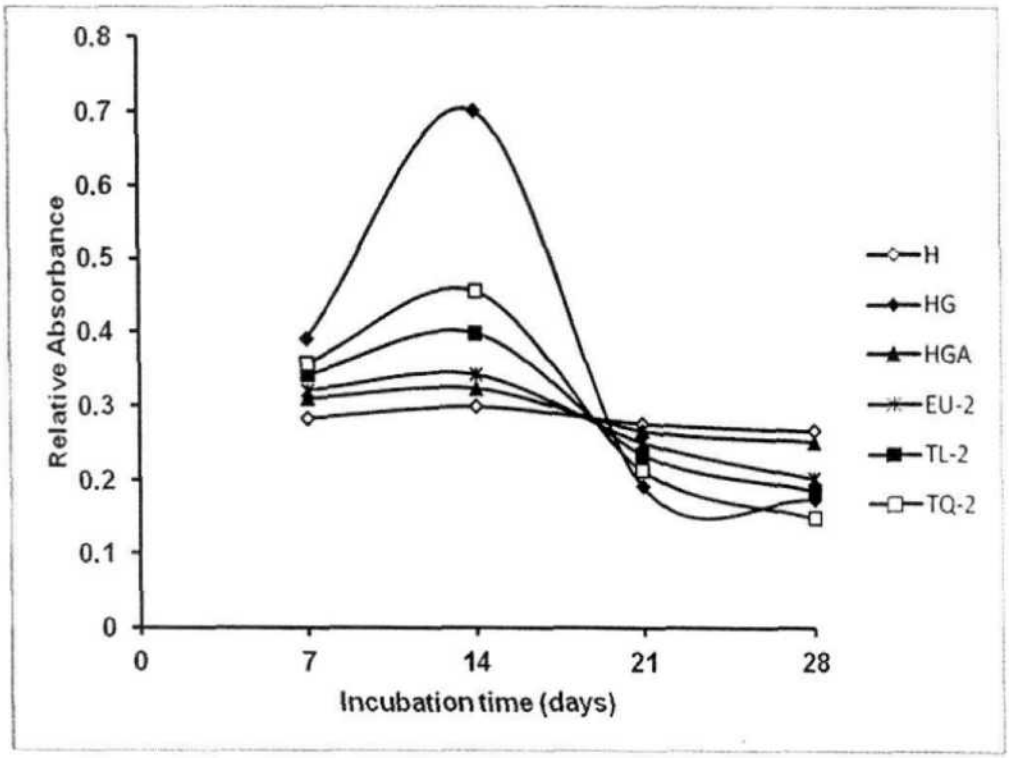

Fig. 64: Relative fluorescence intensity at 335 nm of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) on 7th, 14th, 21st and 28th day of incubation.

Table 6 Percent gain and loss in the tryptophan fluorescence intensity of glycated HSA samples incubated with or without TQ, TL or EU after 7th, 14th, 21st and 28th day of incubation.

Sample	Percent gain in F.I. at 335 nm		Percent loss in F.I. at 335 nm	
	7 days	14 days	21 days	28 days
HG	8.24 %	18.63	48.49	55.81
HGA	1.34 %	5.97	8.57	10.16
TQ-1	6.01 %	14.93	38.16	48.68
TQ-2	5.99 %	14.42	33.95	42.59
TQ-3	9.74 %	16.16	41.49	55.52
TL-1	5.67 %	11.57	28.27	39.61
TL-2	5.38 %	10.49	24.09	32.56
TL-3	6.08 %	13.78	34.78	45.82
EU-1	4.1 %	10.17	15.59	24.9
EU-2	3.62 %	7.93	13.36	20.11
EU-3	5.08 %	12.25	22.65	48.68

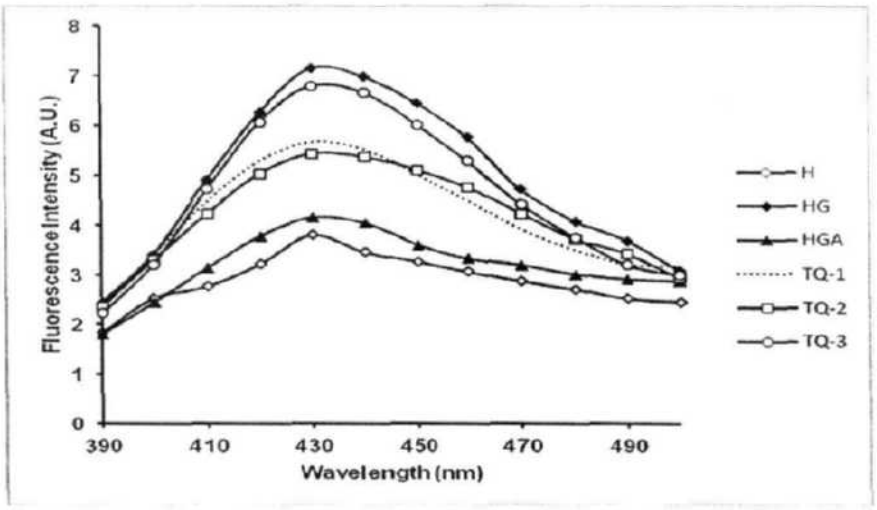

Fig. 65: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 14 days.

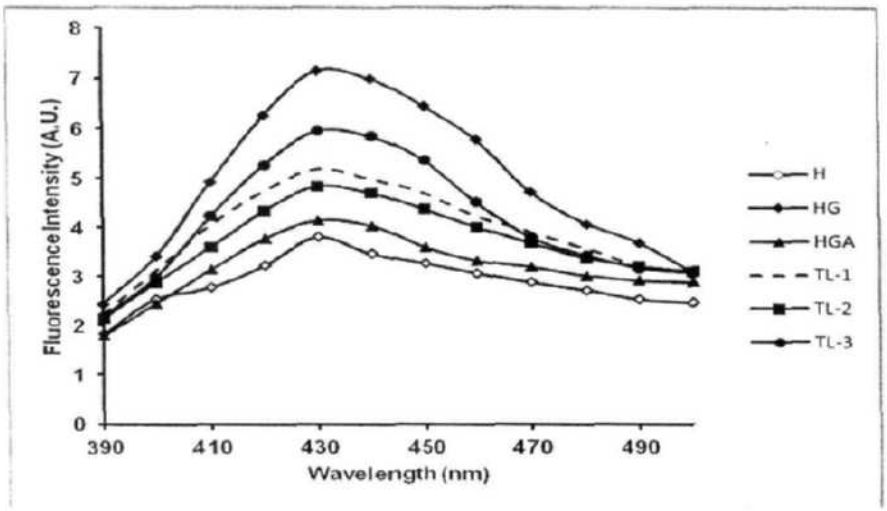

Fig. 66: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 14 days.

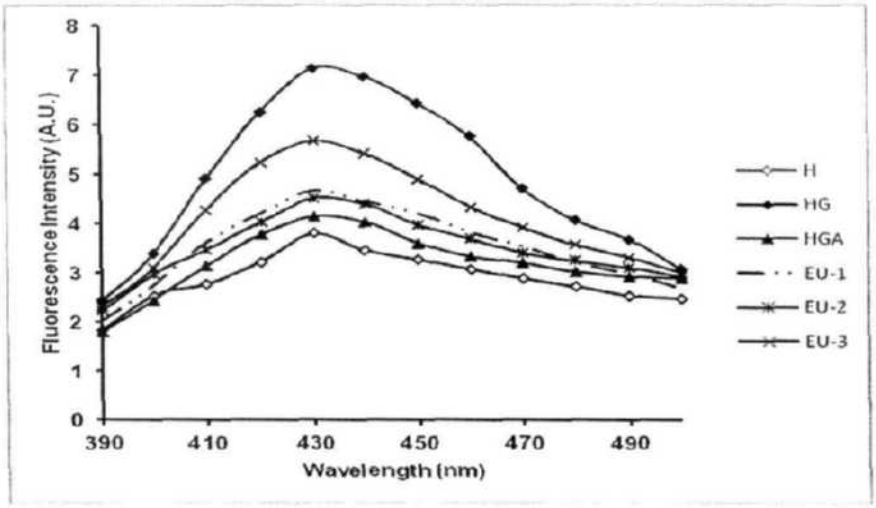

Fig. 67: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 14 days.

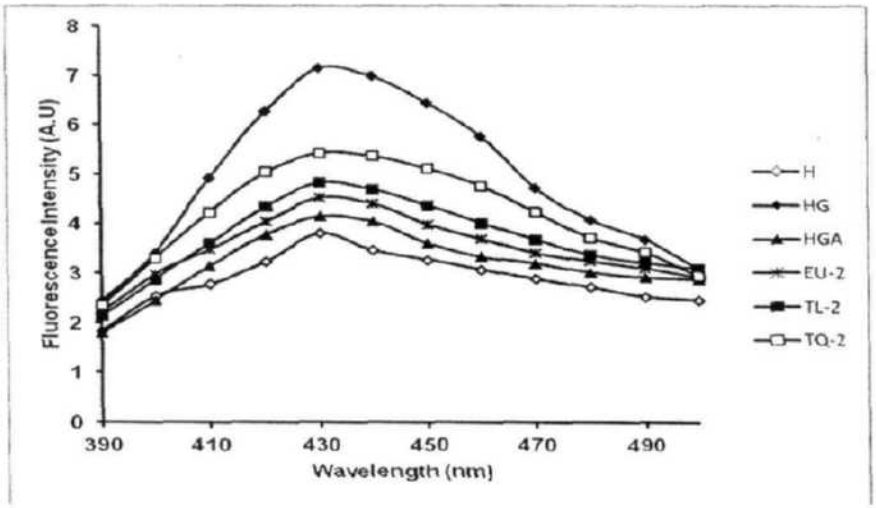

Fig. 68: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 14 days.

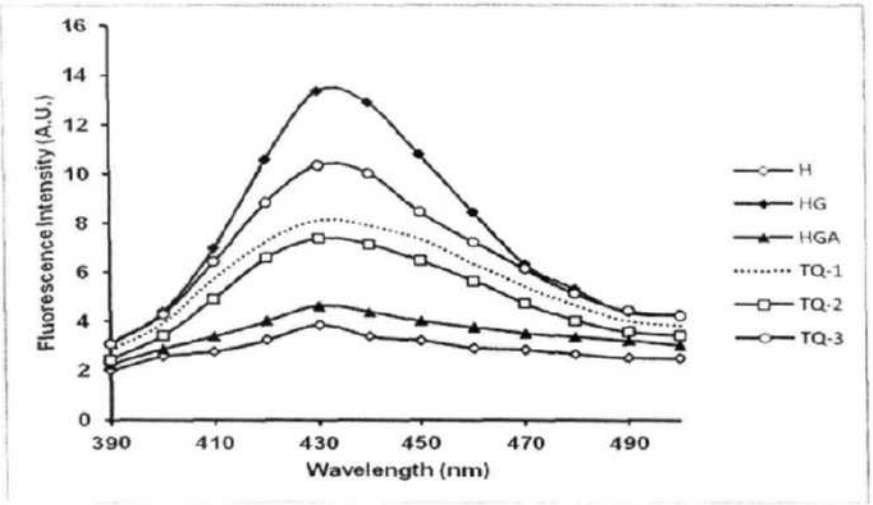

Fig. 69: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 21 days.

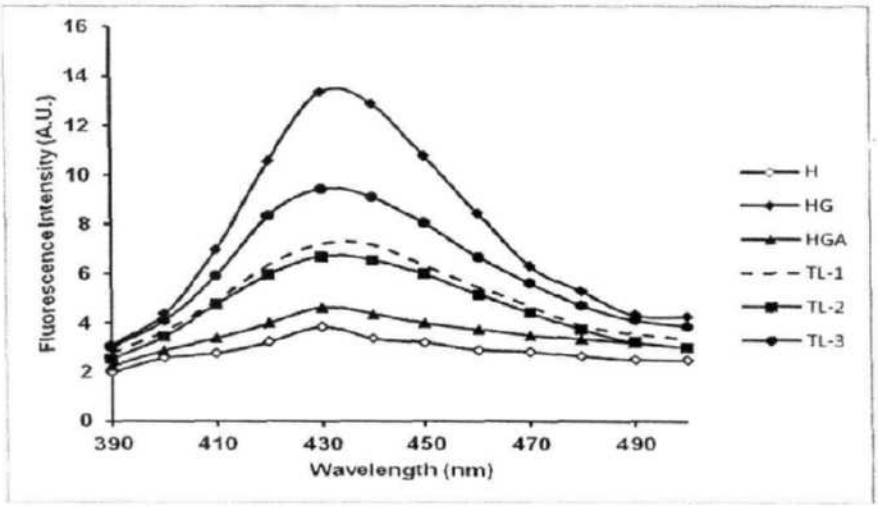

Fig. 70: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 21 days.

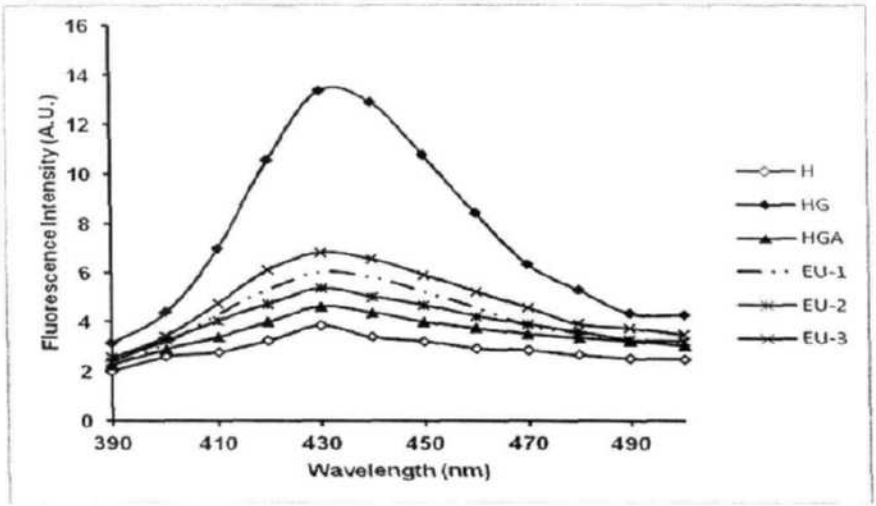

Fig. 71: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 21 days.

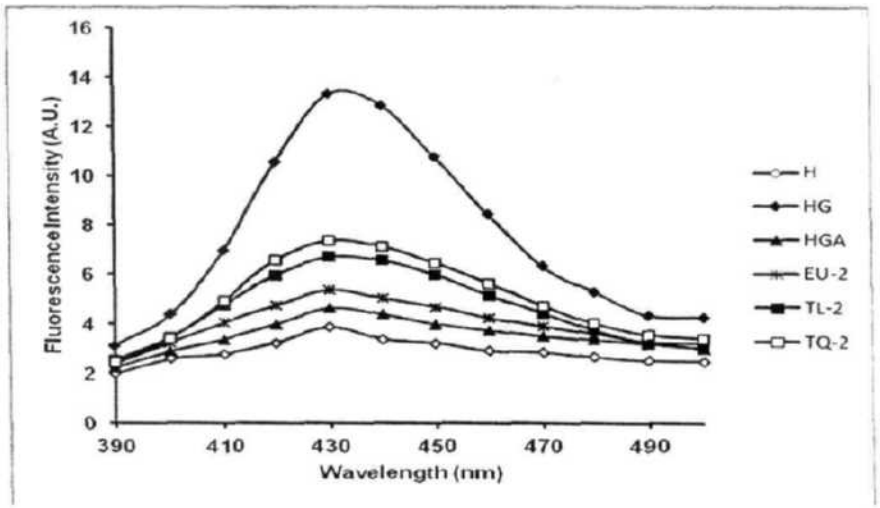

Fig. 72: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) after incubation for 21 days.

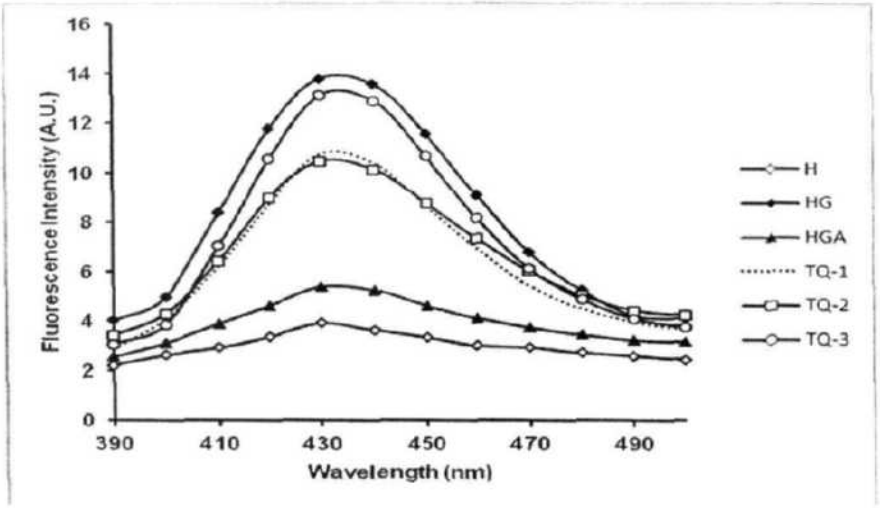

Fig. 73: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TQ-1, TQ-2 and TQ-3 after incubation for 28 days.

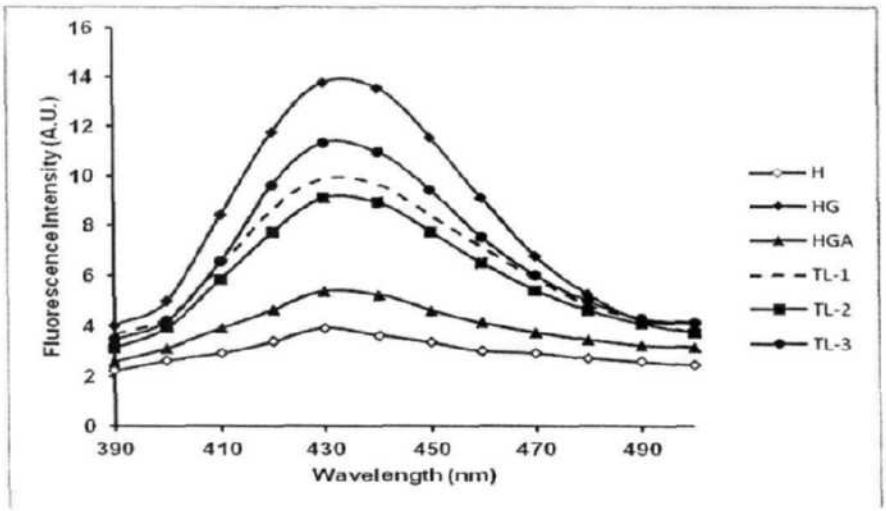

Fig. 74: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), TL-1, TL-2 and TL-3 after incubation for 28 days.

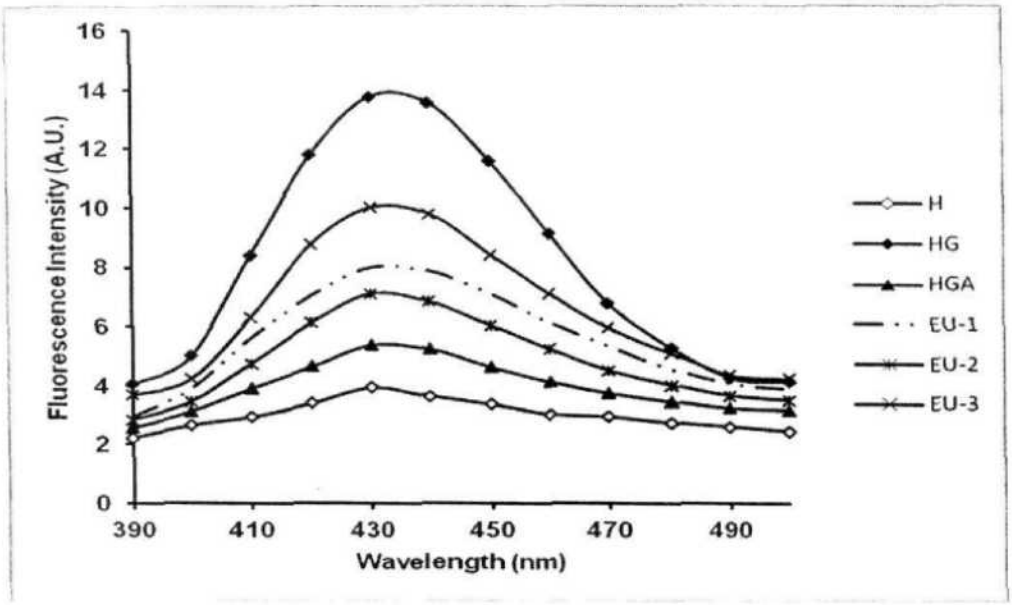

Fig. 75: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), EU-1, EU-2 and EU-3 after incubation for 28 days.

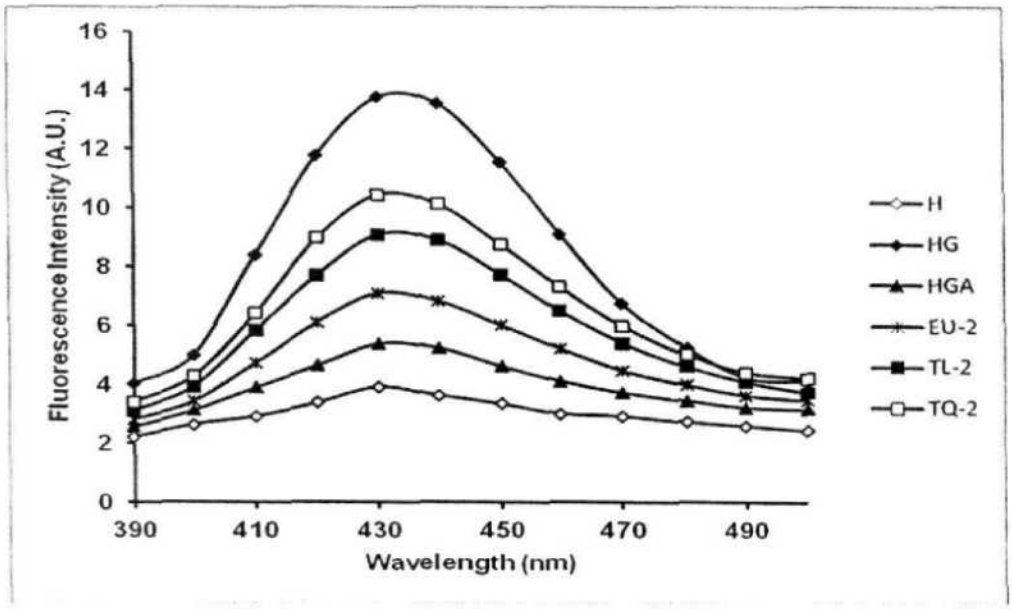

Fig. 76: AGE specific fluorescence spectra of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (T 2) and Eugenol (EU-2) after incubation for 28 days.

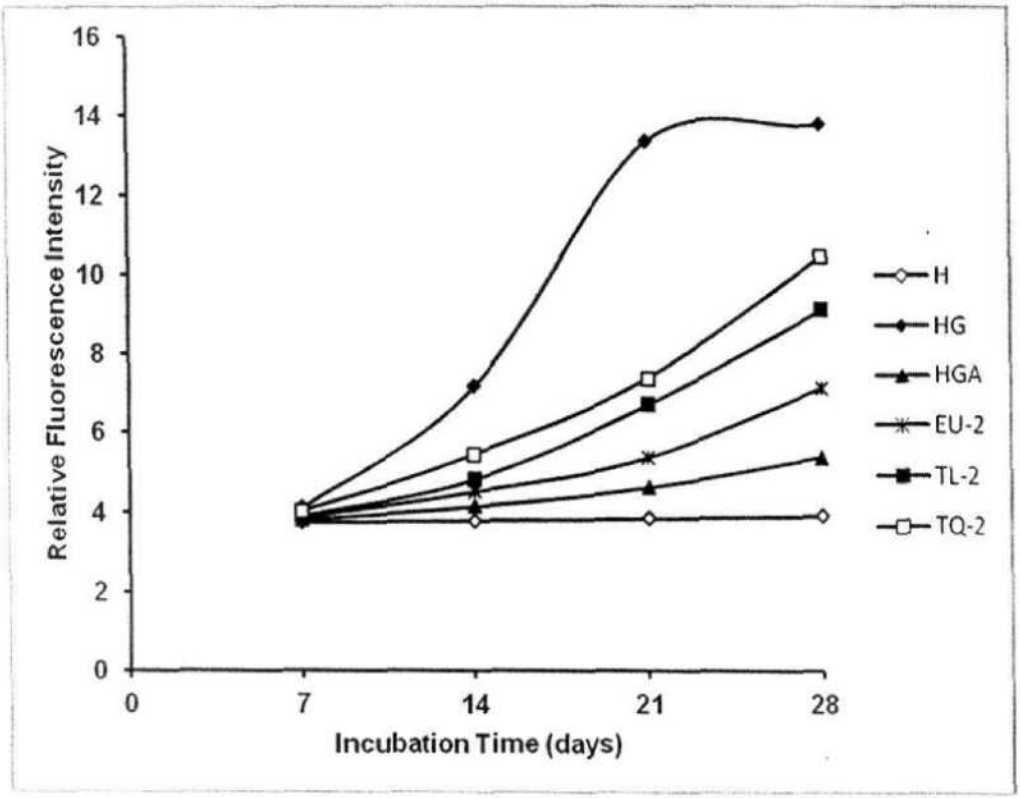

Fig. 77: Relative AGE fluorescence intensity at 430 nm of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), Thymoquinone (TQ-2), Thymol (TL-2) and Eugenol (EU-2) on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of incubation.

Table 7: Effects of TQ, TL and EU on AGEs formation in the HSA-glucose reaction *in vitro*.

Sample	Concentration (µM)	Inhibition of AGE formation (%)		
		14 days	21 days	28 days
AG[a]	1000	42.08	65.38	60.96
TQ-1	3	20.67	39.3	21.88
TQ-2	30	24.04	44.96	24.1
TQ-3	300	5.13	22.6	4.82
TL-1	3	27.74	45.88	28.3
TL-2	30	32.49	49.86	33.88
TL-3	300	16.86	29.22	17.66
EU-1	0.06	34.69	54.97	42.03
EU-2	0.6	36.78	59.86	48.4
EU-3	6.0	20.67	48.93	27.35

[a] Aminoguanidine (AG) was used as positive control.

Fourier Transform Infrared Spectroscopy

Addition of aminoguanidine to the reaction result in the shift of amide I peaks to 1623.11 cm^{-1} and the formation of new peak of group NH-CO at 1699.17 cm^{-1}. A peak at 1074.68 cm-1 showed the interaction of HSA with glucose (Fig. 78). Another peak observed at 1238.81 cm^{-1} shows the bond of O-CO. Addition of thymol to the reaction results in formation of new peak at 1058.67 cm^{-1} and 1090.70 cm^{-1}. Addition of thymoquinone to the reaction showed a shift in peak of epoxy ring and observed at 1178.67 cm^{-1}. Spectra recorded on addition of eugenol to the reaction may leads to vibration stretch in epoxy ring of HSA with peak observed at 1142.81 cm^{-1} (Fig. 78).

Effect of TQ, TL and EU on the level of ketoamines in glycated HSA

The data show ketoamine formation in nmoles/mg of protein in various HSA samples viz. Native HSA, glycated HSA and aminoguanidine (AG), thymoquinone, thymol and eugenol treated HSA incubation with sugar for 7 days (Table 8). The glycated albumin treated with aminoguanidine exhibited a significant decrease in ketoamine level followed by EU-1 and EU-2 treated glycated albumin as compared to glycated HSA. AG had 12.3 ± 1.89 and EU-1 and EU-2 had 19.6 ± 1.92 and 14.5 ± 3.77 in contrast to glycated HSA which had a very high ketoamine conc. of 61.1 ± 2.68 (Fig. 79c). TQ and TL treated glycated albumin also showed decrease in ketoamine levels but not parallel to those observed for AG treated albumin (Fig. 79a and b). Out of the three TQ, TL and EU formulations taken TQ-2, TL-2 and EU-2 showed the highest reduction in ketoamine levels amongst themselves. Ketoamine reduction induced by TQ-2 (28.2 ± 3.31) was followed by TQ-1 (35.3 ± 3.11) and TQ-3 (48.9 ± 2.07); TL-2 (24.6 ± 2.84) was followed by TL-1 (32.7 ± 2.48) and TL-3 (42.4 ± 3.07) and EU-2 (14.5 ± 3.77) was followed by EU-1 (19.6 ± 1.92) and EU-3 (24.4 ± 2.82). Amongst the TQ-2, TL-2 and EU-2 preparations, EU-2 was found to induce highest reduction in ketoamine formation (Fig. 79d). This indicates a very high potency of eugenol as compared to thymoquinone and thymol in decreasing ketoamine levels since it performed better at a concentration of 0.6 µM than 30 µM of either TQ or TL.

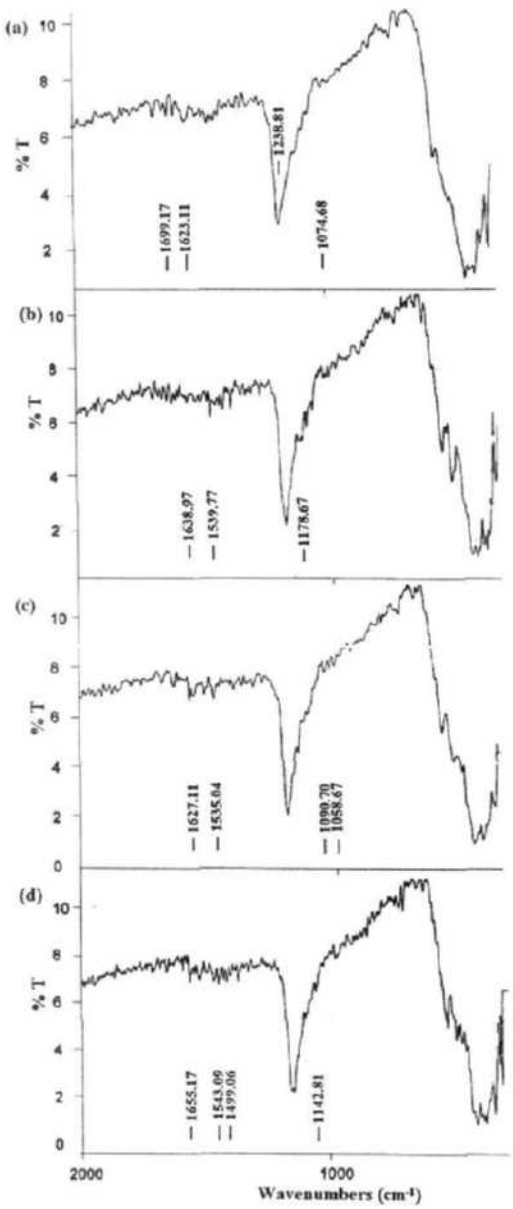

Fig. 78: FTIR spectra of (a) HSA + Glucose + Aminoguanidine (AG); (b) HSA + Glucose + Thymoquinone (TQ); (c) HSA + Glucose + Thymol (TL); and (d) HSA + Glucose + Eugenol (EU).

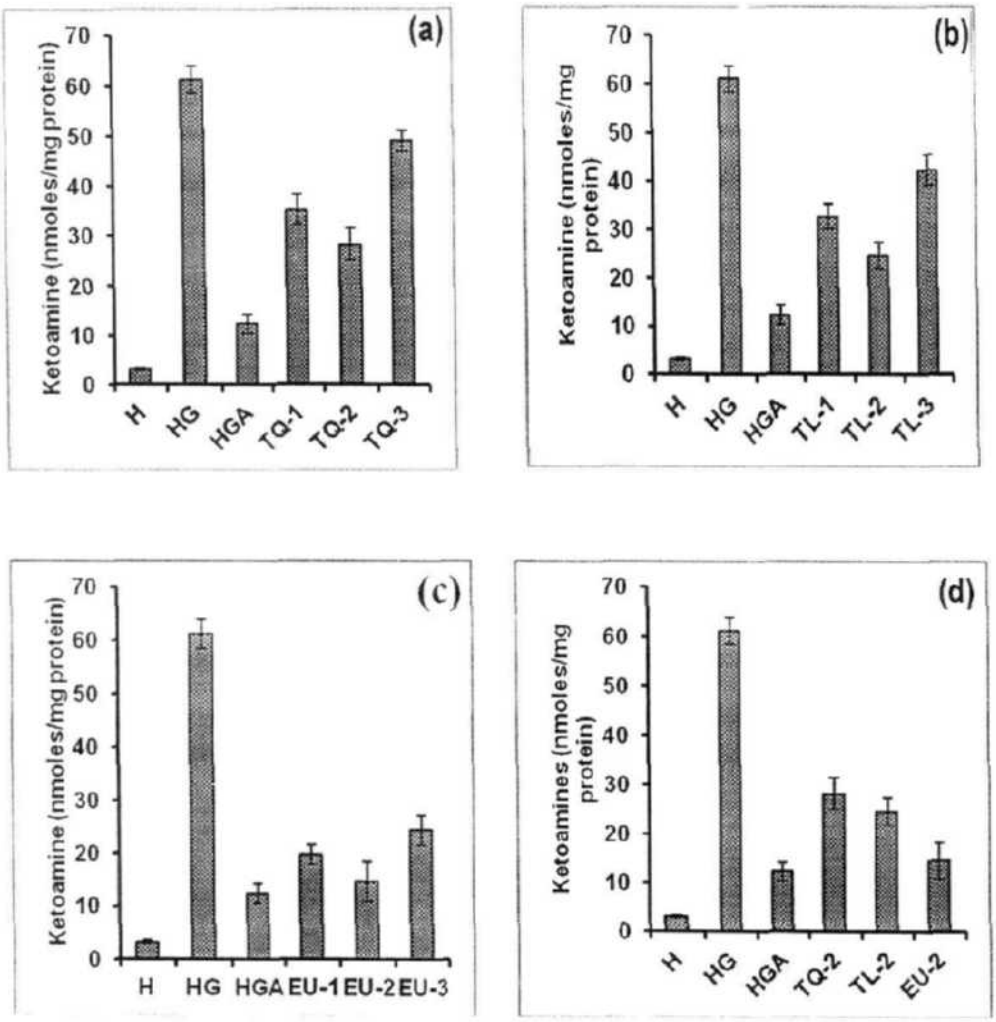

Fig. 79: (a) Level of ketoamines in native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA), varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) at 7th day. Each value represent the means ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

Table 8: Level of ketoamines in native HSA (H), glycated HSA (HG) and glycated HSA after being treated with aminoguanidine (HGA) or varying concentrations of thymoquinone (TQ-1, TQ-2, TQ-3), thymol (TL-1, TL-2, TL-3) and eugenol (EU-1, EU-2, EU-3) on 7 days.

Sample	Concentration (μM)	Ketoamine formation (nmoles/mg of protein)
H	-	3.14 ± 0.26
HG	-	61.1 ± 2.68
AG	1000	12.3 ± 1.89
TQ-1	3	35.3 ± 3.11
TQ-2	30	28.2 ± 3.31
TQ-3	300	48.9 ± 2.07
TL-1	3	32.7 ± 2.48
TL-2	30	24.6 + 2.84
TL-3	300	42.4 ± 3.07
EU-1	0.06	19.6 ± 1.92
EU-2	0.6	14.5 ± 3.77
EU-3	6.0	24.4 ± 2.82

All the data has been represented as Mean ± SD for three experiments performed in duplicates.

Significantly different at $p<0.05$.

Comparison of the inhibitory effect of phytochemicals (TQ, TL and EU) at various concentrations on glycation-induced HSA oxidation

The protein carbonyl level which is an assessment of protein oxidation was also determined for various HSA preparations at various time points 7, 14, 21 and 28 post glycation. As expected, protein oxidation markedly increased upon glycation of HSA (Fig. 28, Table 9). AG at a concentration of 1mM was found to exhibit highest reduction in HSA oxidation at all the time points as compared to other agents taken. TQ at a concentration of 30 μM showed a good decrease in protein carbonyl levels at each time point and interestingly with increase in time period the gap in the protein carbonyl levels widened. Both 3 μM and 300 μM of TQ although could induce reduction in HSA oxidation but not as effectively as TQ-2 (Fig. 80a). TL formulations were found to perform better in decreasing protein carbonyl levels as compared to TQ preparations. EU formulations at all the three concentrations and at all the time points were found to induce better reduction than the corresponding TQ and TL formulations (Fig. 80-83). Parallel to the ketoamine results, EU-2 i.e. 0.6 μM of eugenol was found to reduce the carbonyl content better than TQ-2 and TL-2 (30 μM of thymiquinone and thymol) after 7, 14, 21 and 28 days of incubation with sugar (Fig. 84).

A comparative study of the percent decrease in free amino groups of glycated HSA treated with various concentrations of phytochemicals

Glycated HSA treated with various concentrations of phytochemicals for 1-4 weeks was also studied for loss of free amino groups. In concordance with earlier results, AG was found to lower the loss of free amino groups of glycated albumin significantly as compared to TQ, TL and EU at all the time points although each group of preparation could induce some reduction in loss of free amino groups (Fig. 85-88). TQ-2 (30 μM) as observed for earlier studies, was found to decrease the loss of free amino groups better than the two other formulations. Similarly, 30 μM of TL i.e. TL-2 could lead to reduction in percent decrease of free amino group more than TL-1 and TL-3 after each incubation period. All the EU formulations induced more reduction in loss of free amino groups than their corresponding TQ and TL formulations (Table 10) and in concordance with earlier results EU-2 was found to be better in decreasing the loss of free amino groups than TQ-2 and TL-2 at all the time points (Fig. 89).

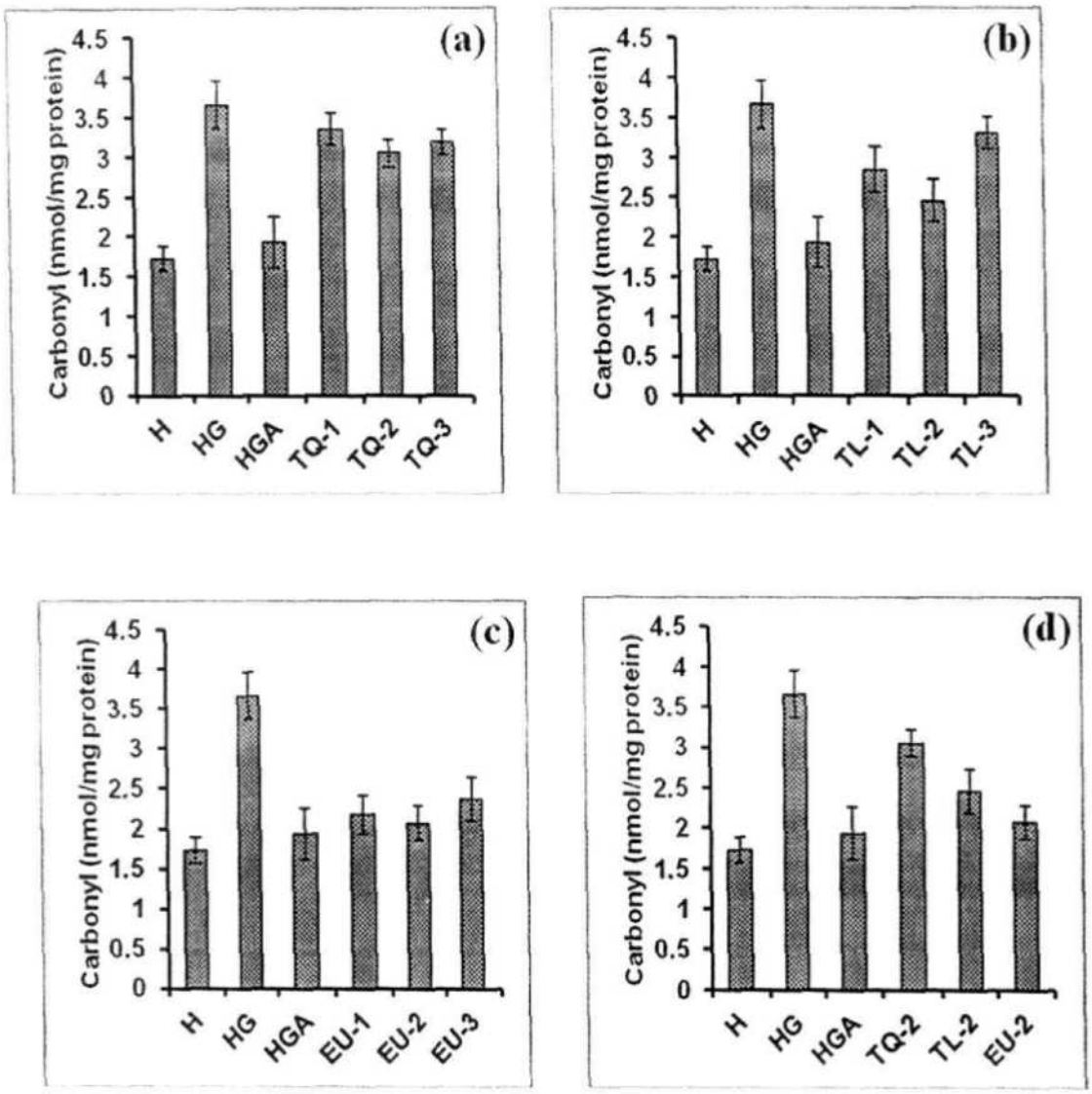

Fig. 80: Carbonylation of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 µM (TQ-2), thymol-30 µM (TL-2) or eugenol-0.6 µM (EU-2) on 7 days. Each value represent the Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

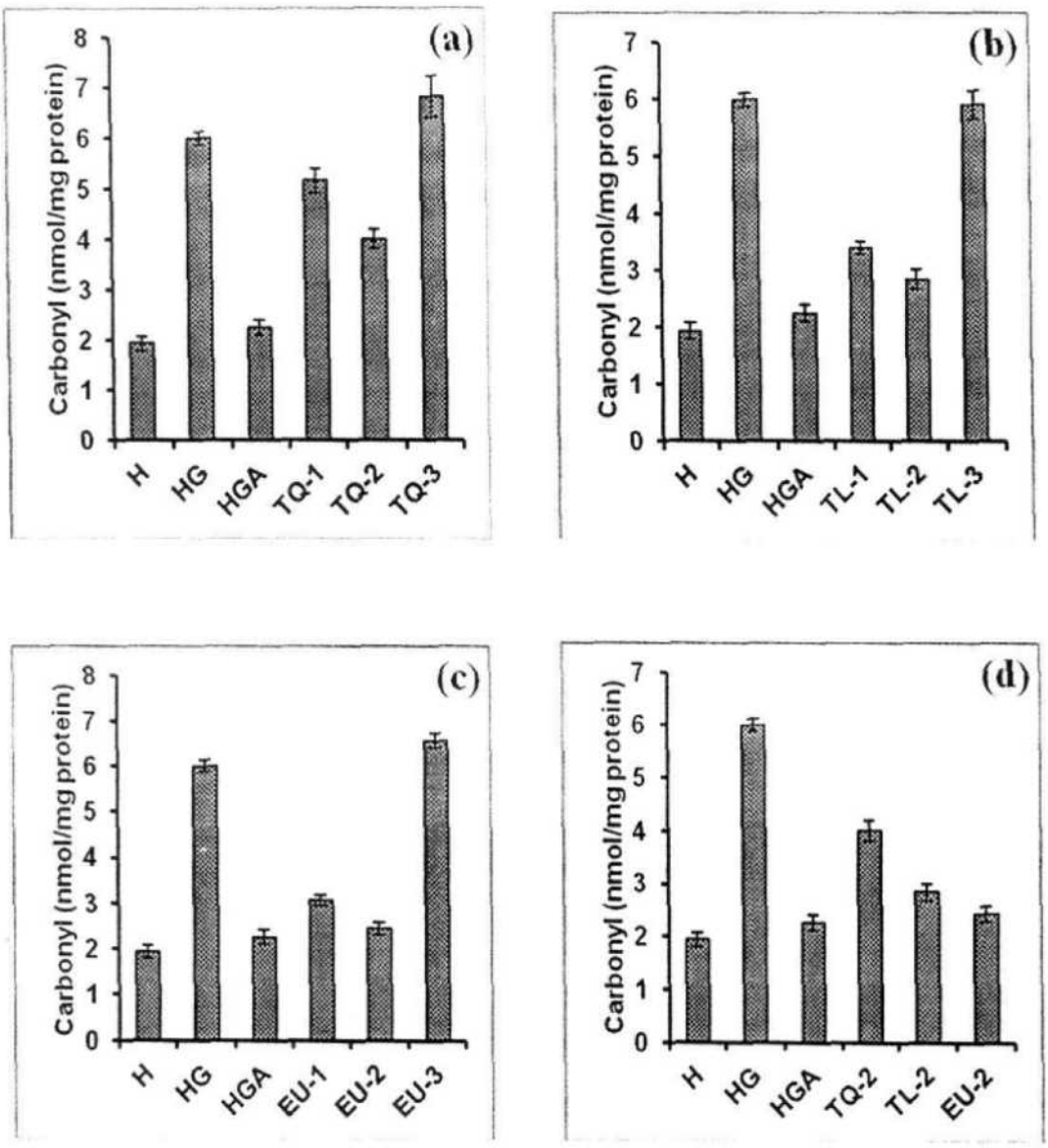

Fig. 81: Carbonylation of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) on 14 days. Each value represent the Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

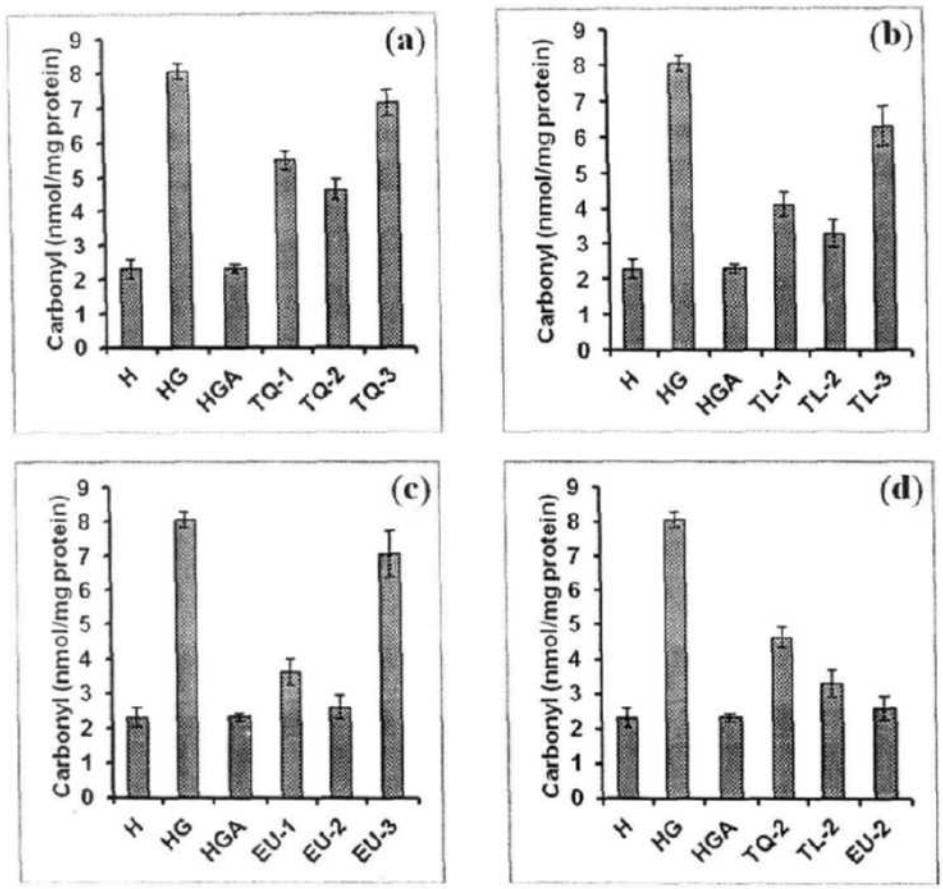

Fig. 82: Carbonylation of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 µM (TQ-2), thymol-30 µM (TL-2) or eugenol-0.6 µM (EU-2) on 21 days. Each value represent the Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

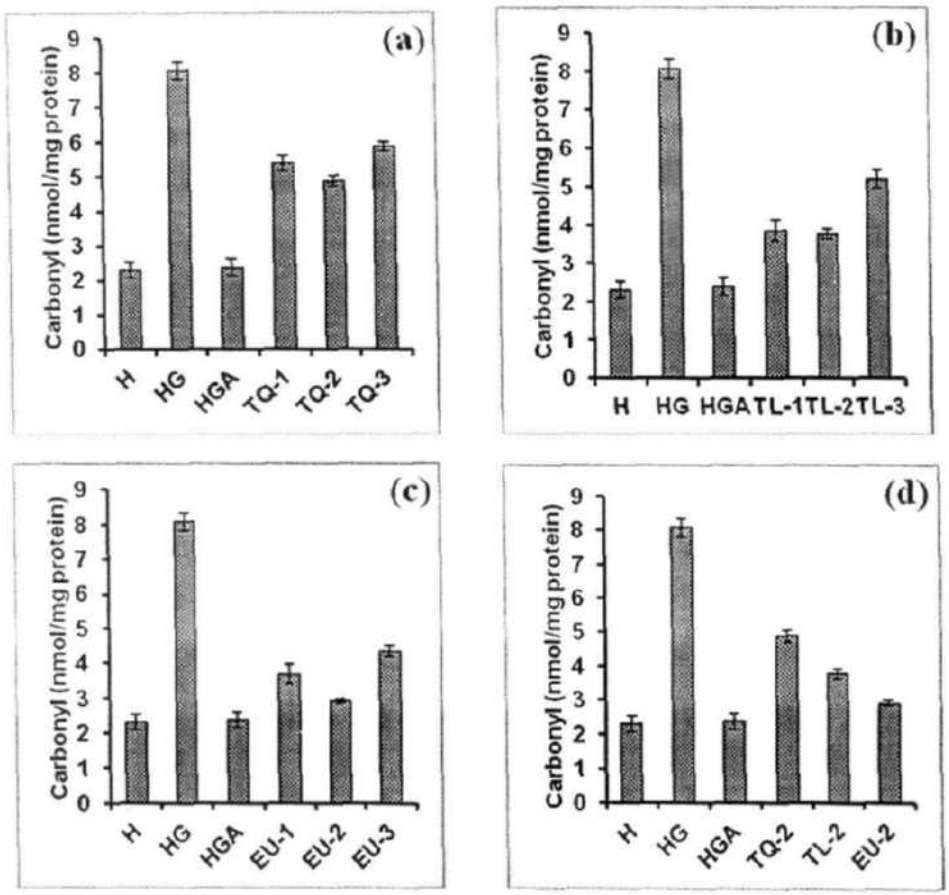

Fig. 83: Carbonylation of native HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) on 28 days. Each value represent the Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

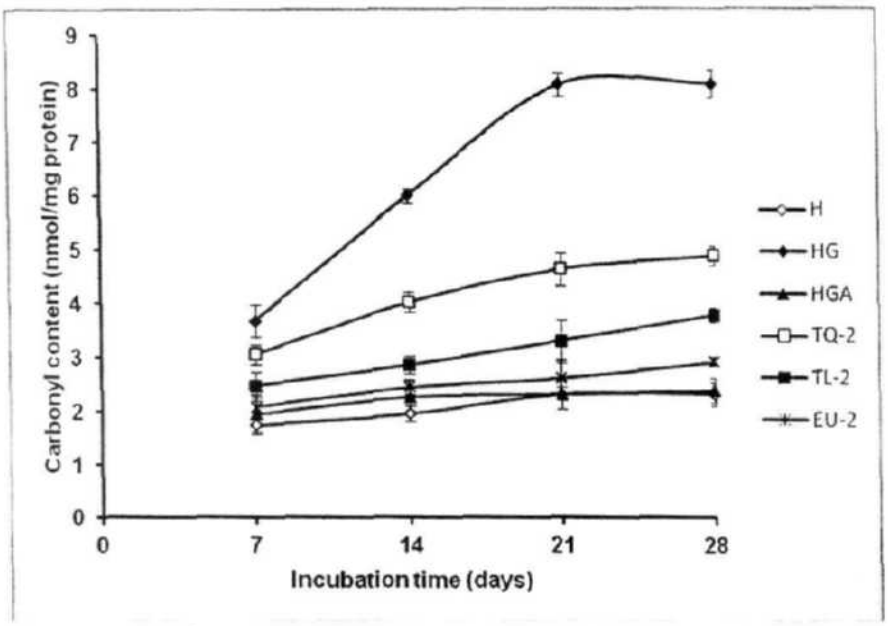

Fig. 84: Carbonylation of HSA incubated with 100 mM glucose in absence and presence of aminoguanidine (HGA), thymoquinone-30 µM (TQ-2), thymol-30 µM (TL-2) or eugenol-0.6 µM (EU-2) up to 28 days. HSA incubated under identical conditions without any treatment act as control. Each value represent the Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

Table 9: Protein Carbonyl content (nmoles /mg protein) present in HSA samples incubated with glucose and varying concentrations of TQ, TL or EU at 7, 14, 21 and 28 days.

Sample	7 days	14 days	21 days	28 days
H	1.73 ± 0.58	1.94 ± 0.14	2.30 ± 0.27	2.30 ± 0.22
HG	3.66 ± 0.30	5.99 ± 0.13	8.06 ± 0.22	8.07 ± 0.26
HGA	1.94 ± 0.32	2.26 ± 0.15	2.31 ± 0.12	2.37 ± 0.23
TQ-1	3.35 ± 0.2	5.17 ± 0.25	5.48 ± 0.27	5.39 ± 0.23
TQ-2	3.05 ± 0.17	4.01 ± 0.19	4.63 ± 0.3	4.87 ± 0.17
TQ-3	3.18 ± 0.16	6.82 ± 0.40	7.13 ± 0.36	5.87 ± 0.12
TL-1	2.85 ± 0.29	3.40 ± 0.12	4.12 ± 0.34	3.85 ± 0.28
TL-2	2.46 ± 0.26	2.85 ± 0.16	3.29 ± 0.4	3.77 ± 0.14
TL-3	3.3 ± 0.21	5.91 ± 0.24	6.31 ± 0.54	5.19 ± 0.25
EU-1	2.18 ± 0.24	3.07 ± 0.10	3.62 ± 0.39	3.66 ± 0.28
EU-2	2.073 ± 0.21	2.43 ± 0.14	2.6 ± 0.33	2.90 ± 0.08
EU-3	2.37 ± 0.27	6.57 ± 0.15	7.05 ± 0.68	4.35 ± 0.16

All the data has been represented as Mean ± SD for three experiments performed in duplicates.

Significantly different at $p<0.05$.

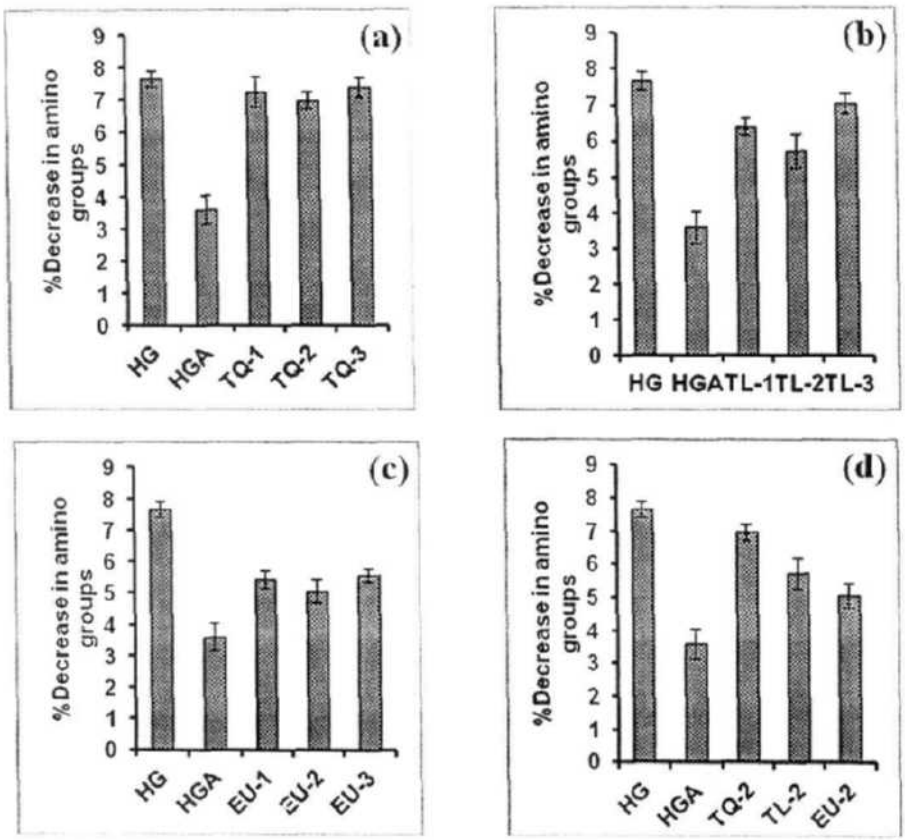

Fig. 85: (a) Percent decrease in amino groups of glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) on 7 days. All the data has been represented as Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$

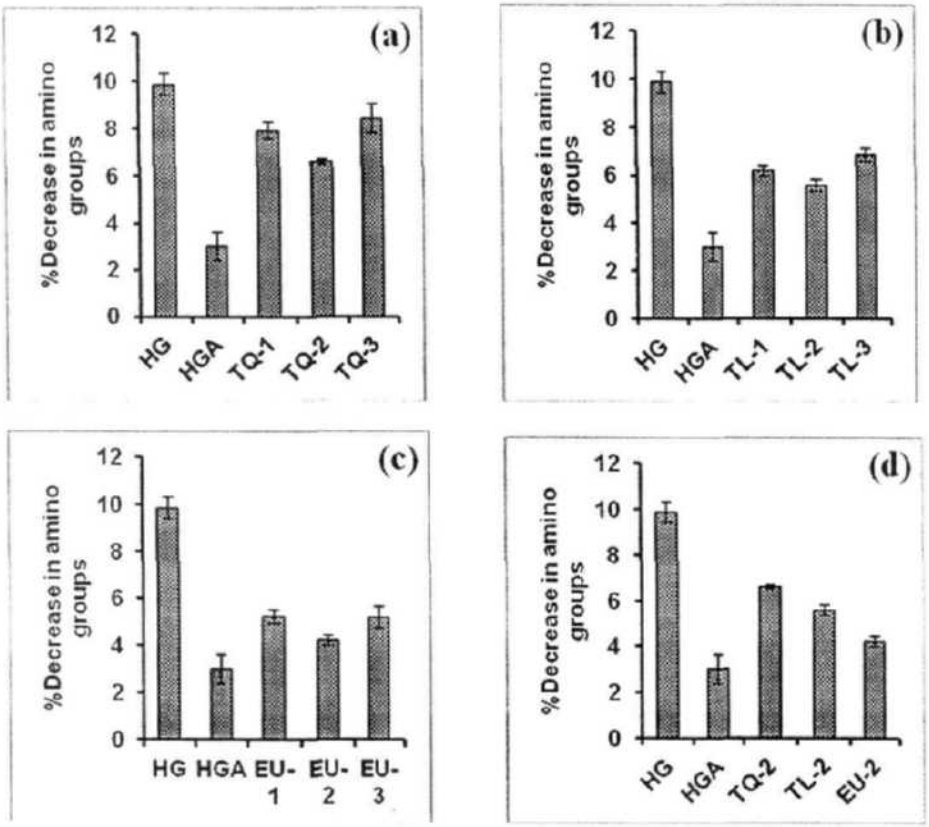

Fig. 86: (a) Percent decrease in amino groups of glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) on 14 days. All the data has been represented as Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$

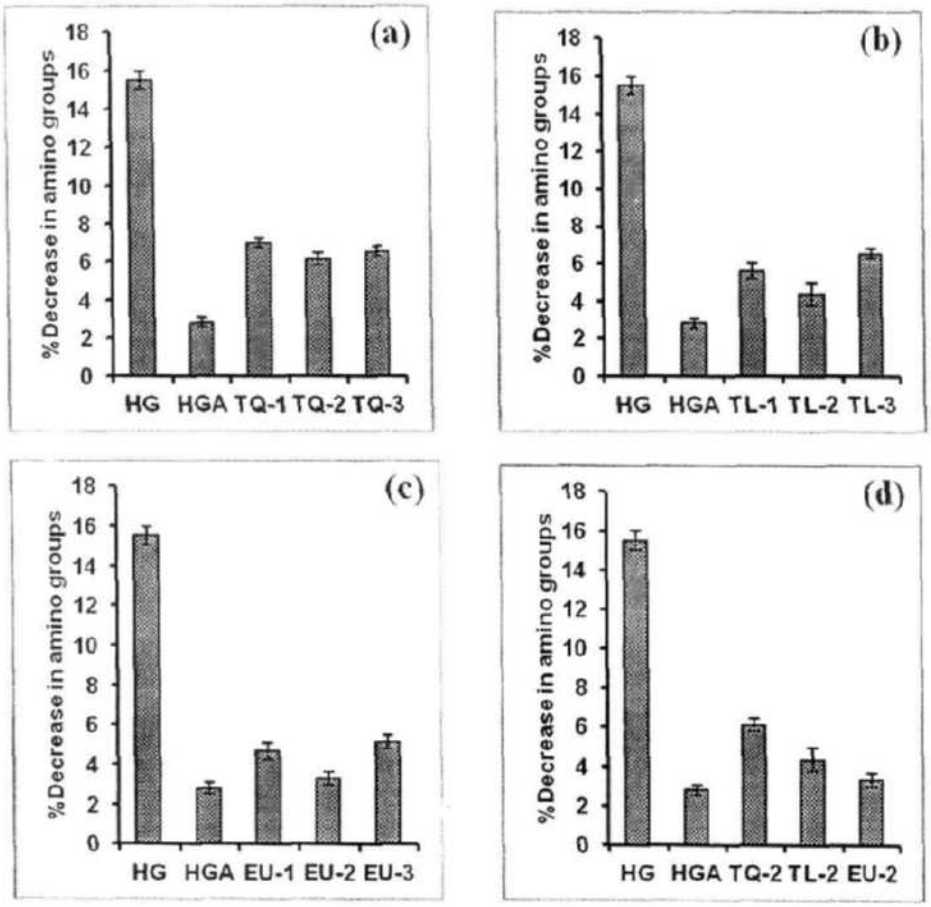

Fig. 87: (a) Percent decrease in amino groups of glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 µM (TQ-2), thymol-30 µM (TL-2) or eugenol-0.6 µM (EU-2) on 21 days. All the data has been represented as Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$

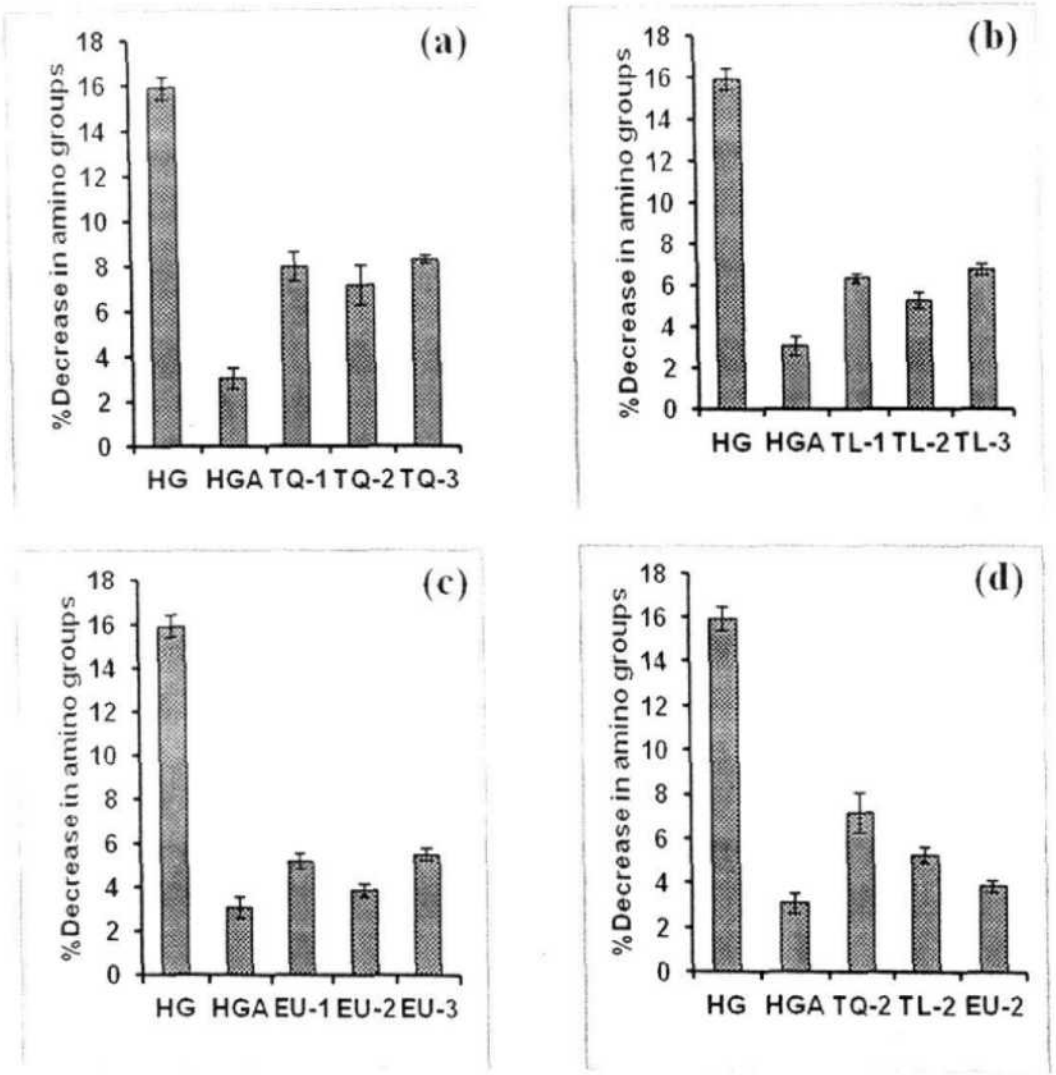

Fig. 88: (a) Percent decrease in amino groups of glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3; (b) varying concentrations of thymol i.e, TL-1, TL-2 and TL-3; (c) varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3. (d) thymoquinone-30 μM (TQ-2), thymol-30 μM (TL-2) or eugenol-0.6 μM (EU-2) on 28 days. All the data has been represented as Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$

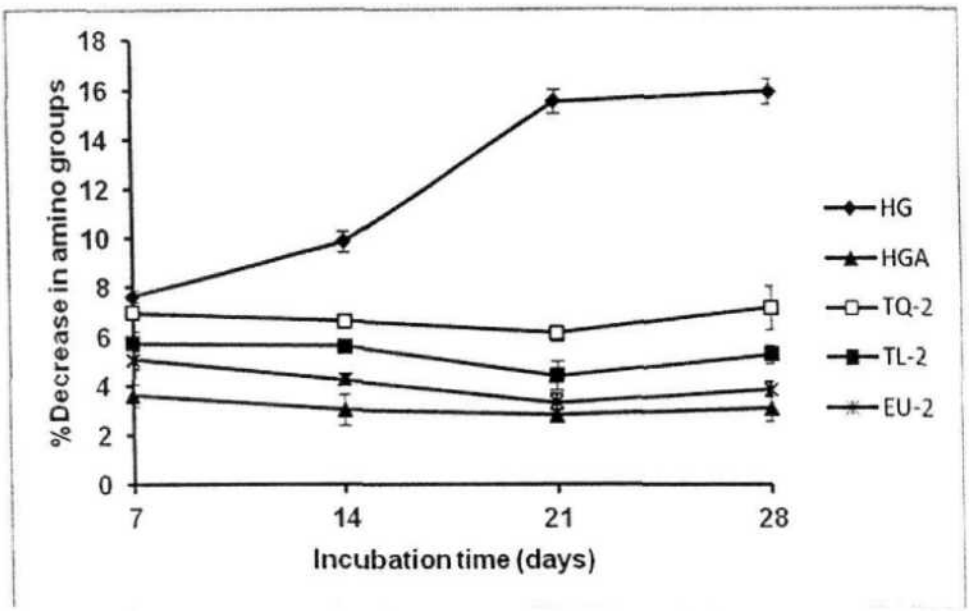

Fig. 89: Percent decrease in amino groups of HSA incubated with 100 mM glucose in absence and presence of aminoguanidine (HGA), thymoquinone (TQ-2, 30 μM), thymol-30 μM (TL-2, 30 μM) or eugenol (EU-2, 0.6 μM) up to 28 days. All the data has been represented as Mean ± SD for three experiments performed in duplicates. Significantly different at $p<0.05$.

Table 10: Percent decrease in free amino groups of glycated HSA samples incubated in absence and presence of varying concentrations of TQ, TL or EU at 7, 14, 21 and 28 days.

Sample	7 days	14 days	21 days	28 days
HG	7.65 ± 0.24	9.87 ± 0.44	15.51 ± 0.47	15.91 ± 0.52
HGA	3.60 ± 0.45	3.01 ± 0.62	2.81 ± 0.26	3.05 ± 0.47
TQ-1	7.24 ± 0.46	7.93 ± 0.36	6.96 ± 0.23	7.97 ± 0.65
TQ-2	6.96 ± 0.26	6.6 ± 0.11	6.14 ± 0.32	7.16 ± 0.90
TQ-3	7.39 ± 0.31	8.41 ± 0.62	6.52 ± 0.27	8.28 ± 0.16
TL-1	6.39 ± 0.22	6.18 ± 0.22	5.62 ± 0.44	6.34 ± 0.21
TL-2	5.73 ± 0.47	5.59 ± 0.23	4.38 ± 0.58	5.24 ± 0.38
TL-3	7.03 ± 0.28	6.84 ± 0.27	6.55 ± 0.27	6.74 ± 0.26
EU-1	5.42 ± 0.27	5.23 ± 0.31	4.69 ± 0.42	5.18 ± 0.35
EU-2	5.04 ± 0.37	4.23 ± 0.21	3.31 ± 0.34	3.83 ± 0.29
EU-3	5.56 ± 0.2	5.19 ± 0.47	5.15 ± 0.32	5.48 ± 0.26

All the data has been represented as Mean ± SD for three experiments performed in duplicates.

Significantly different at $p<0.05$.

Effect of phytochemicals on superoxide anion radical generation

To confirm that ROS are involved in the observed propagation of protein damage, we measured generation of $O_2^{\bullet-}$ from glycated HSA. Superoxide dismutase (SOD) was used as the standard quencher of $O_2^{\bullet-}$ (Fig. 90) and showed significant inhibition in superoxide radical formation in glycated HSA. The amount of $O_2^{\bullet-}$ was found to be 21.7 ± 1.23 nmol/ml in glycated HSA. However, addition of AG induced reduction in $O_2^{\bullet-}$ generation to 5.4 ± 1.09 nmol/ml. Out of the three TQ, TL and EU formulations taken, TQ-2, TL-2 and EU-2 showed the highest reduction in $O_2^{\bullet-}$ formation amongst themselves. Generation of superoxide anion radical reduced by TQ-2 (8.8 ± 0.69) was followed by TQ-1 (9.2 ± 1.75) and TQ-3 (18.8 ± 0.93); TL-2 (7.4 ± 1.44) was followed by TL-1 (7.7 ± 0.78) and TL-3 (16.7 ± 1.39) and EU-2 (5.9 ± 0.69) was followed by EU-1 (6.7 ±1.18) and EU-3 (16.5 ± 1.42) (Fig.91). EU-2 i.e. 0.6 µM of eugenol was found to decrease the $O_2^{\bullet-}$ formation better than TQ-2 and TL-2 (30 µM of thymoquinone and thymol) (Fig.91d).

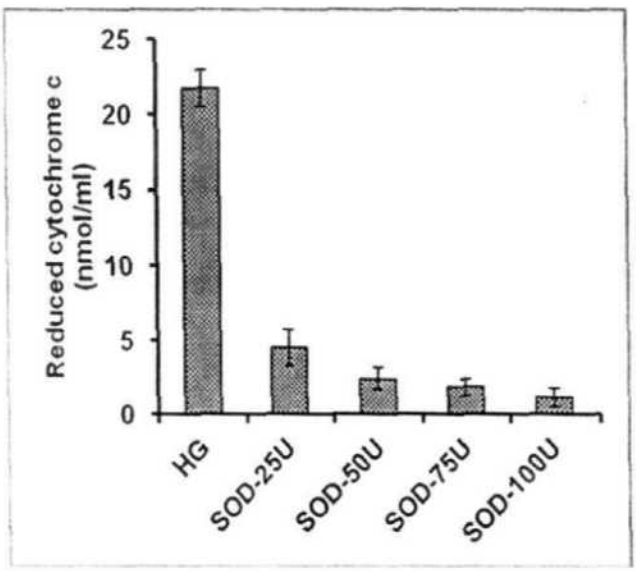

Fig. 90: Inhibition of superoxide radical generation during glycation of HSA by superoxide dismutase.

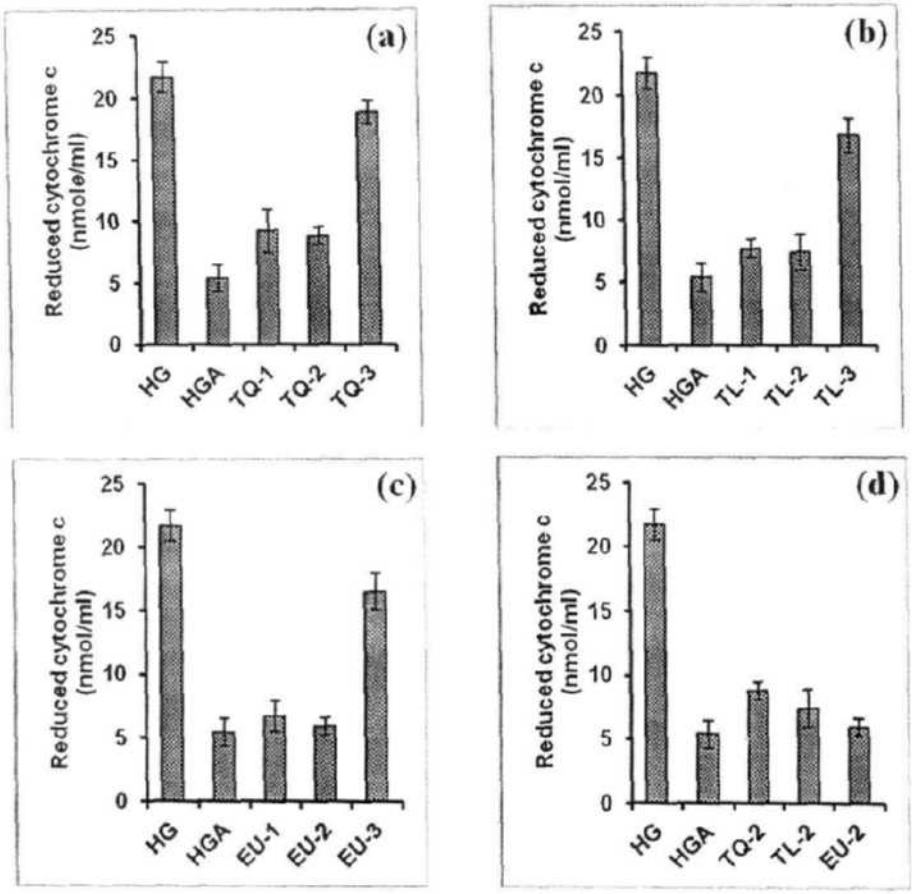

Fig. 91: (a) Superoxide anion radical generation on incubation of HSA with glucose (HG) and its inhibition on treatment of glycated HSA with aminoguanidine (HGA) and varying concentrations of thymoquinone; (b) Inhibition of $O_2^{\bullet-}$ generation on treatment with varying concentrations of thymol; (c) Inhibition of $O_2^{\bullet-}$ generation on treatment with varying concentrations of eugenol and (d) Comparison between best concentration TQ-2, TL-2 and EU-2 in inhibiting $O_2^{\bullet-}$ generation.

Effect of TQ, TL and EU on electrophoretic pattern of HSA in-vitro

Hyperglycaemia, the major complication of diabetes, leads to protein glycation and formation of advanced glycation end products. Amadori products and glucose in the glycated protein undergo oxidation to form free radicals which can induce protein fragmentation. Phytochemicals thymoquinone (TQ), thymol (TL) and eugenol (EU) have been attributed with potent antioxidant property. Human Serum Albumin (HSA) was glycated in the presence of different concentrations of phytochemicals viz. TQ, TL and EU and their effect on protein fragmentation inhibition was examined by sodium dodecyl sulphate ployacrylamide gel electrophoresis (SDS-PAGE).

HSA when incubated with glucose undergoes protein cross-linking and aggregate formation. Crosslink inhibition of glycated HSA post incubation with various preparations of phytochemicals viz. TQ, TL and EU was observed at various time points (7 days, 14 days, 21 days and 28 days). HSA incubated with glucose (Fig. 92, lane 3) exhibits slight fragmentation and hence intensity of band reduces with diffusion which is not observed for HSA incubated alone (Fig. 92, lane 2). Aminoguanidine at a concentration of 1 mM significantly inhibited the HSA crosslinks. All the three preparations of phytochemical EU showed inhibition in aggregate formation but EU-2 (0.6 μM) was found to be the best inhibitor amongst its group at all the time points although it could not achieve the level observed for AG inhibition (Fig. 98, 99; lane 4). EU-1 was found to inhibit fragmentation better than EU-3 after incubation periods 7, 14, 21 and 28 days (Fig. 98, 99). Similar case was observed for TQ (Fig. 92, 93) and TL (Fig. 95, 96) phytochemicals. Amongst the three formulations of TL and TQ, TL-2 and TQ-2 were found to inhibit HSA crosslink formation and/or fragmentation better than other formulations of their group after each incubation period. As observed in Fig. 100, EU-2 significantly inhibited diffusion of glycated HSA band due to protein cross-links induced by protein glycation in comparison to TL-2 and TQ-2.

Densitometric analysis

SDS-PAGE was also analyzed by gel densitometry. Density of a portion of band of equal area was found to be decreased on glycation of HSA. However, on treatment with phytochemicals, density of gel increased showing the restriction of cross-links and prevented HSA from glycation (Table 11). EU-2 inhibited protein fragmentation to a great extent. Relative density was found to be 605.8, 604.2, 590.6 and 581.0 at 7, 14, 21 and 28 days of EU-2 incubation which is much closer to density of native HSA (608.6) (Fig. 100). EU-1 showed higher density as compared to EU-3 after incubation periods 7, 14, 21 and 28 days (Fig. 100). Similar case was observed for TL and TQ phytochemicals. Amongst the three formulations of TL and TQ, TL-2 and TQ-2 were found to have greater density which is in relation to PAGE analysis of respective groups after each incubation period (Fig. 94, 97). Among the best concentration of phytochemicals, EU-2 showed maximum increase in density (Fig. 102).

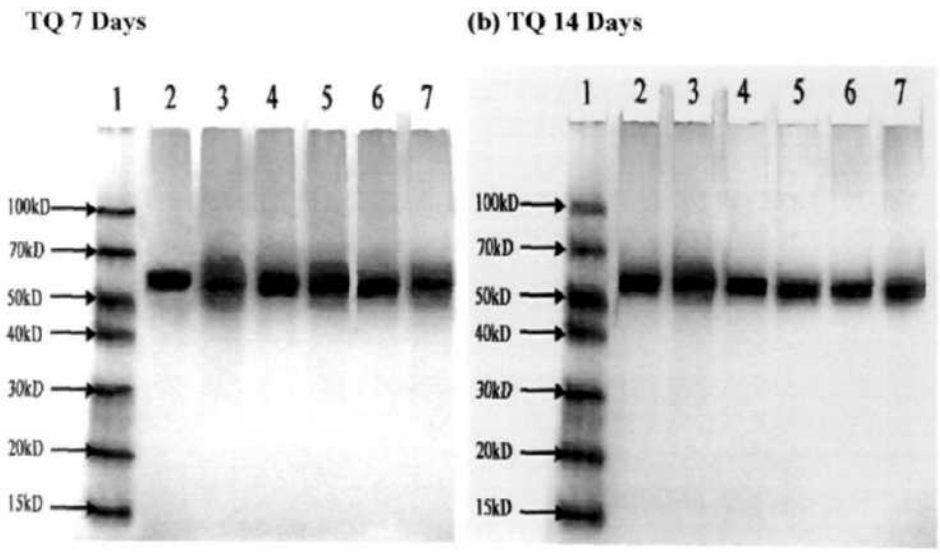

Fig. 92: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of TQ-1, TQ-2 or TQ-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 7 days; (b) 14 days.

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + TQ-1 (3 µM)

Lane 6: HSA + Glucose + TQ-2 (30 µM)

Lane 7: HSA + Glucose + TQ-3 (300 µM)

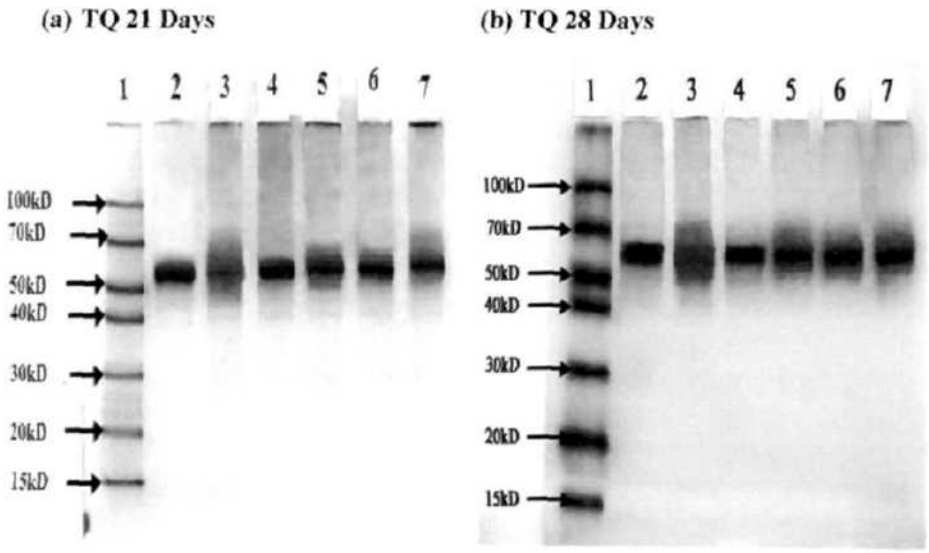

Fig. 93: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of TQ-1, TQ-2 or TQ-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days; (b) 28 days.

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + TQ-1 (3 µM)

Lane 6: HSA + Glucose + TQ-2 (30 µM)

Lane 7: HSA + Glucose + TQ-3 (300 µM)

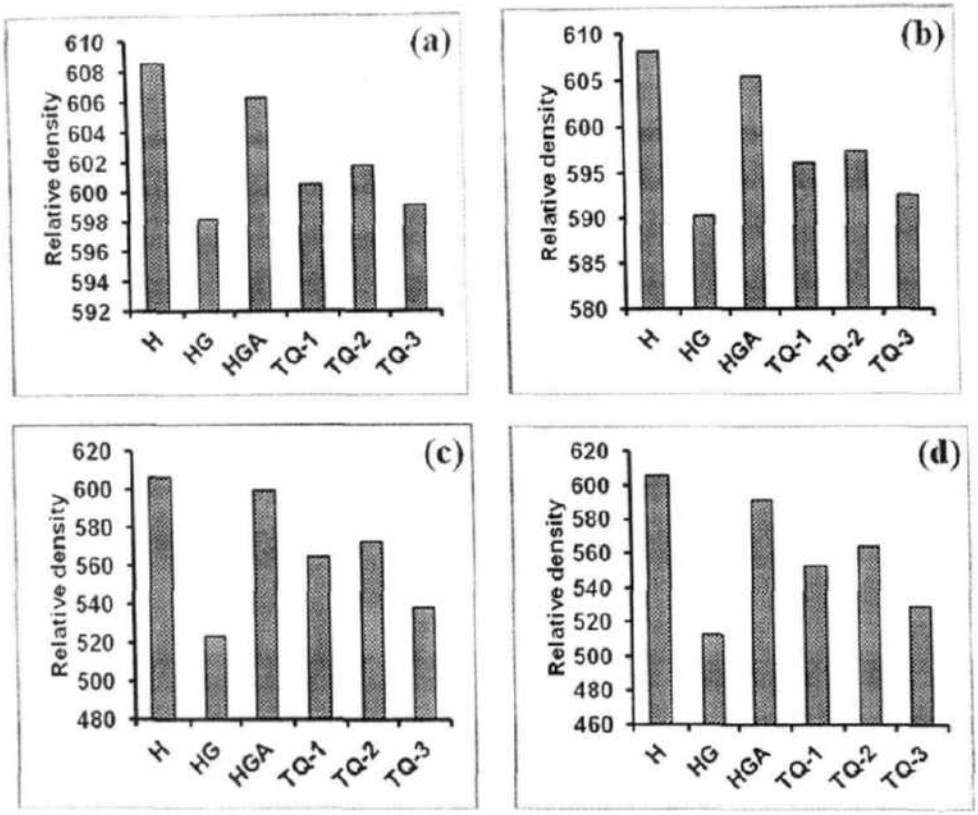

Fig. 94: Densitometric analysis of SDS-PAGE. (a) Histograms represent relative mean band density of HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymoquinone i.e, TQ-1, TQ-2 and TQ-3 at 7 days; (b) 14 days; (c) 21 days and (d) 28 days.

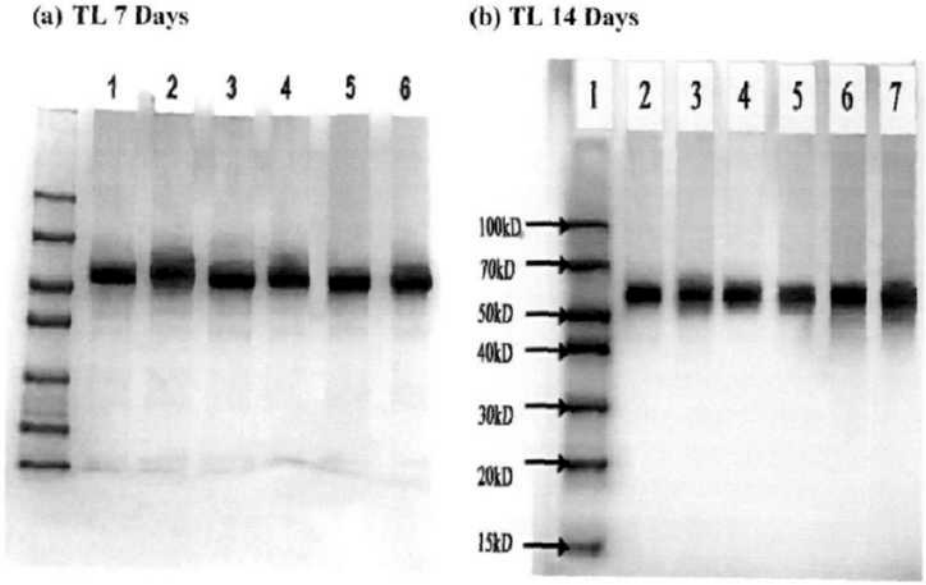

Fig. 95: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of TL-1, TL-2 or TL-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 7 days; (b) 14 days.

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + TL-1 (3 µM)

Lane 6: HSA + Glucose + TL-2 (30 µM)

Lane 7: HSA + Glucose + TL-3 (300 µM)

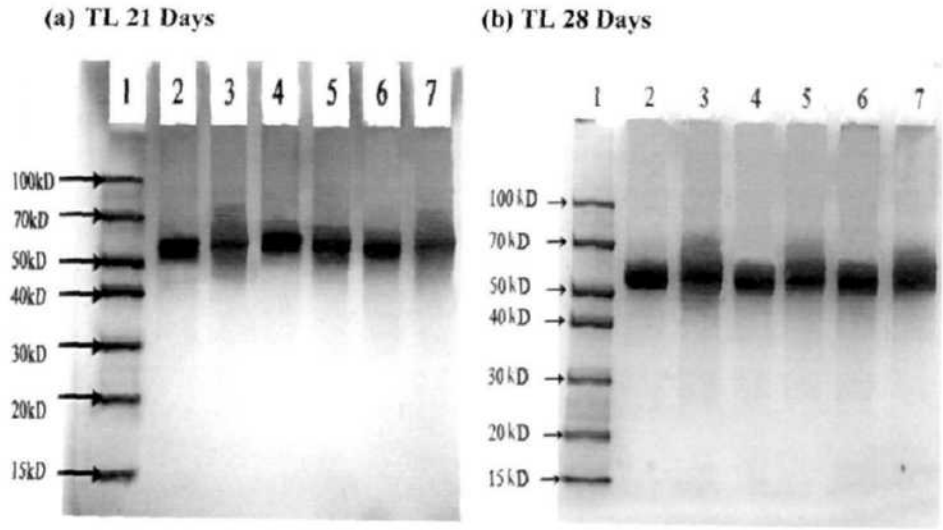

Fig.96: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of TL-1, TL-2 or TL-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days; (b) 28 days.

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + TL-1 (3 µM)

Lane 6: HSA + Glucose + TL-2 (30 µM)

Lane 7: HSA + Glucose + TL-3 (300 µM)

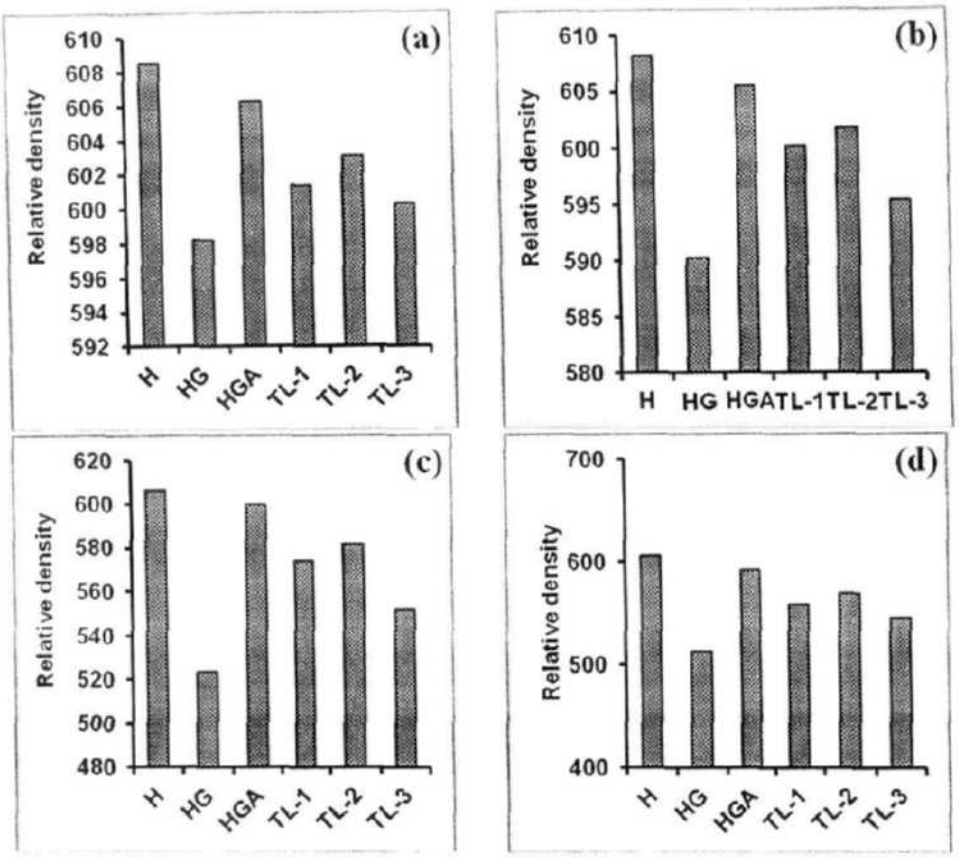

Fig. 97: Densitometric analysis of SDS-PAGE. (a) Histograms represent relative mean band density of HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of thymol i.e, TL-1, TL-2 and TL-3 at 7 days; (b) 14 days; (c) 21 days and (d) 28 days.

(a) EU 7 Days **(b) EU 14 Days**

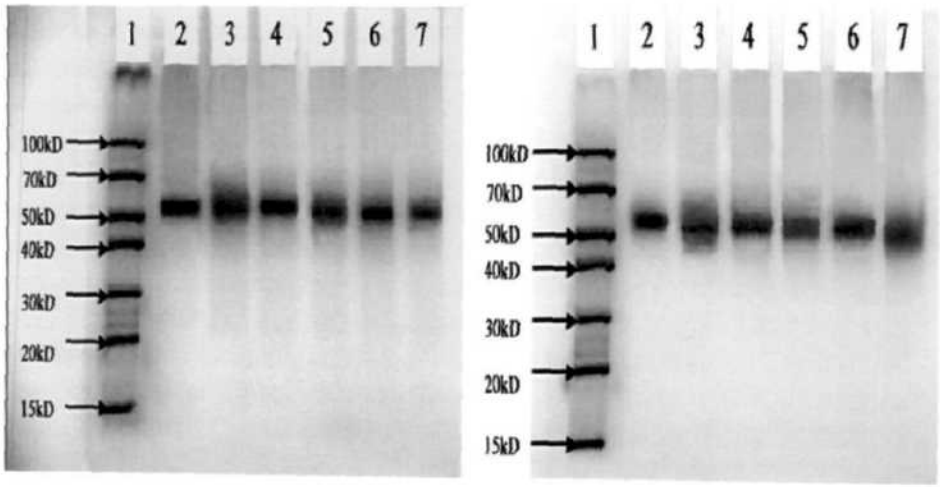

Fig. 98: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of aminoguanidine, EU-1, EU-2 or EU-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 7 days; (b) 14 days

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + EU-1 (0.06 µM)

Lane 6: HSA + Glucose + EU-2 (0.6 µM)

Lane 7: HSA + Glucose + EU-3 (6.0 µM)

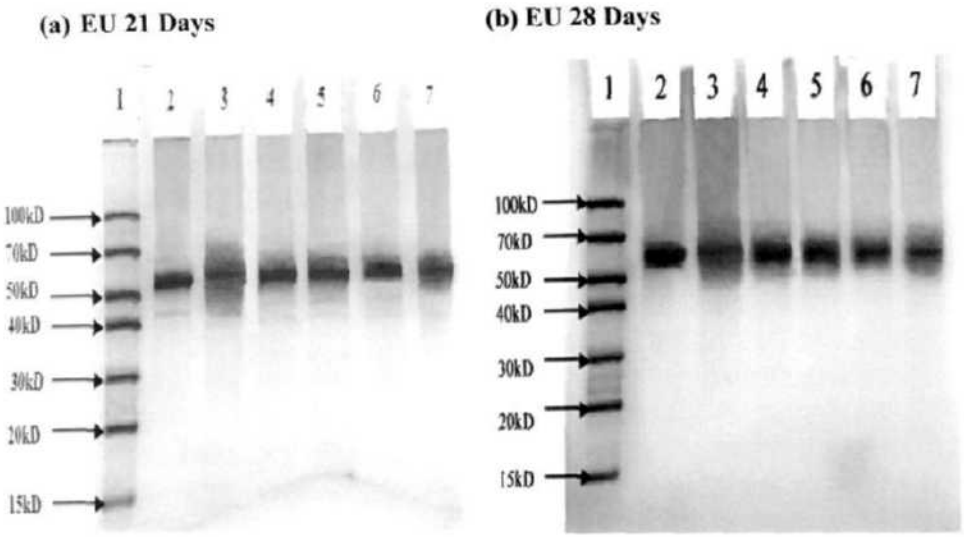

Fig. 99: (a) SDS-PAGE of HSA incubated with glucose in absence and presence of aminoguanidine, EU-1, EU-2 or EU-3 in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days; (b) 28 days

Lane 1: Molecular weight Marker in kD

Lane 2: HSA (10 µg protein)

Lane 3: HSA + Glucose (100 mM)

Lane 4: HSA + Glucose + Aminoguanidine (1 mM)

Lane 5: HSA + Glucose + EU-1 (0.06 µM)

Lane 6: HSA + Glucose + EU-2 (0.6 µM)

Lane 7: HSA + Glucose + EU-3 (6.0 µM)

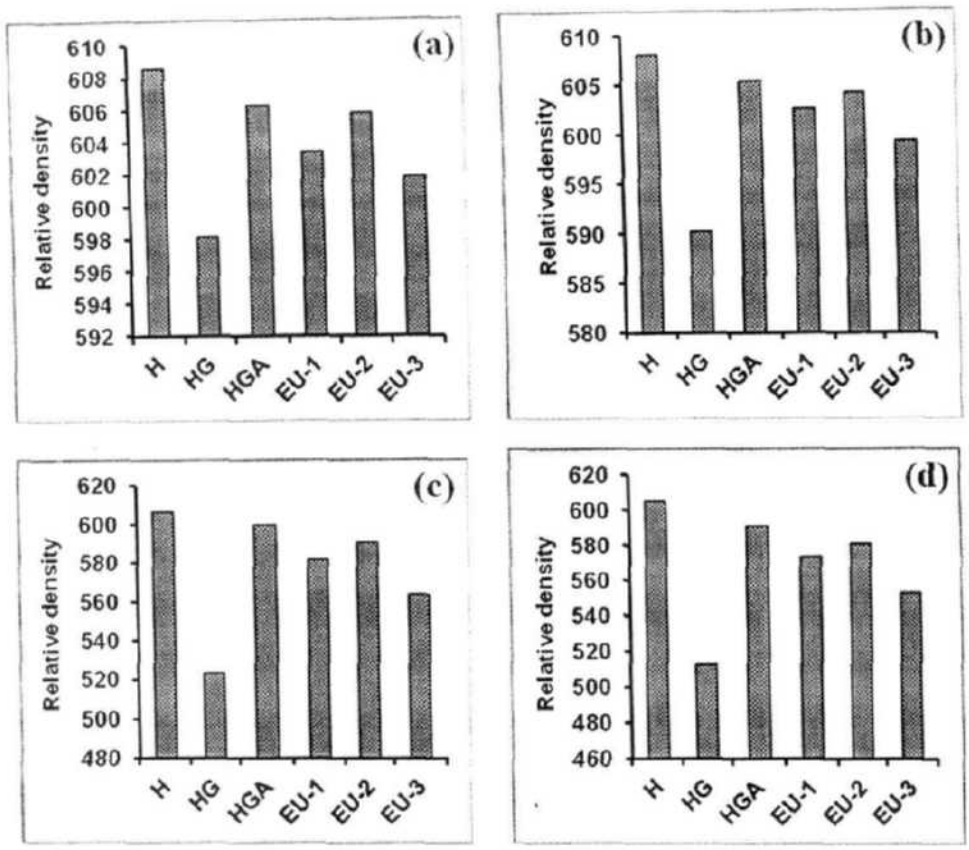

Fig. 100: Densitometric analysis of SDS-PAGE. (a) Histograms represent relative mean band density of HSA (H), glycated HSA (HG) and glycated HSA treated with aminoguanidine (HGA) or varying concentrations of eugenol i.e, EU-1, EU-2 and EU-3 at 7 days; (b) 14 days; (c) 21 days and (d) 28 days.

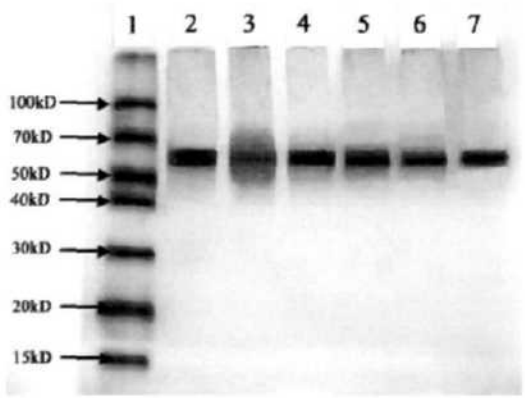

Fig. 101: SDS-PAGE of HSA incubated with glucose (100 mM) in presence of TQ-2, TL-2 or EU-2 in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days.

Lane 1: Molecular weight Marker in kD
Lane 2: HSA (10 µg protein)
Lane 3: HSA + Glucose (100 mM)
Lane 4: HSA + Glucose + Aminoguanidine (1 mM)
Lane 5: HSA + Glucose + TQ-2 (30 µM)
Lane 6: HSA + Glucose + TL-2 (30 µM)
Lane 7: HSA + Glucose + EU-2 (0.6 µM)

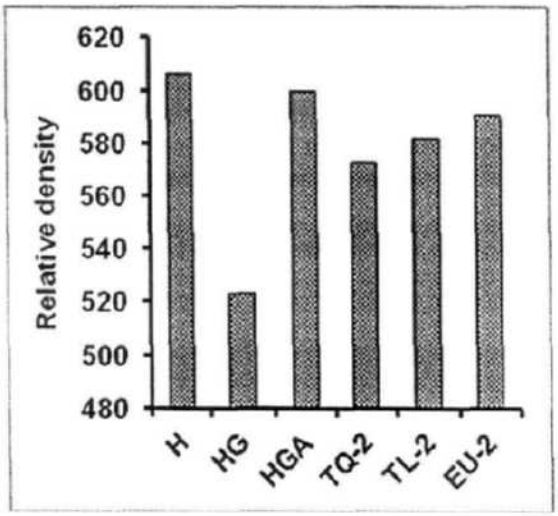

Fig. 102: Comparative densitometric analysis of band density of TQ-2, TL-2 and EU-2 after 21 days of incubation.

Table 11: Densitometric analysis of SDS-PAGE showing effect of varying concentrations of TQ, TL and EU after 7, 14, 21 and 28 days of incubation.

Sample	Relative Density			
	7 days	**14 days**	**21 days**	**28 days**
H	608.6	608.2	606.4	605.7
HG	598.2	590.3	523.2	512.6
HGA	606.3	605.5	599.6	591.1
TQ-1	600.5	596.0	564.7	552.6
TQ-2	601.8	595.2	572.4	563.8
TQ-3	599.1	592.5	538.2	529.1
TL-1	601.4	600.1	574.1	558.4
TL-2	603.1	601.7	581.8	569.3
TL-3	600.3	595.3	551.9	544.7
EU-1	603.4	602.6	581.3	573.9
EU-2	605.8	604.2	590.6	581.0
EU-3	601.8	599.3	563.6	552.7

PROTECTIVE ROLE OF TQ, TL AND EU IN SERA OF TYPE-2 DIABETIC PATIENTS

Effect was observed on sera of type-2 diabetic patients incubated with phytochemicals, viz. TQ, TL and EU and compared with sera incubated in same conditions in absence of phytochemicals for 21 days. Sera of healthy individual without any treatment served as control.

Estimation of malondialdehyde (MDA)

Lipid peroxidation is also a parameter for the evaluation of oxidative stress. Hence, estimation of level of MDA, lipid peroxidation product, in healthy (control) versus diabetic patient's serum was the obvious to investigate. The result depicted in (Fig.103) showed an appreciable augmentation in the MDA levels in diabetic patient's serum (7.01 ± 0.34 nmoles/ml, $p<0.001$) in comparison to control (3.31 ± 0.34 nmoles/ml). Further analysis of diabetic sera incubated with TQ (30 µM), TL (30 µM) and EU (0.6 µM) showed significant decrease in MDA level ($p<0.001$) as compared to diabetic serum without any treatment. As shown in Table 12, the level of MDA in diabetic serum treated with TQ (30 µM), TL (30 µM) and EU (0.6 µM) were found to be 6.02 ± 0.28, 4.87 ± 0.32 and 3.73 ± 0.32 nmoles/ml respectively.

Estimation of carbonyl contents

Carbonyl content was determined to assess the glycoxidative stress induced protein oxidation. Our observation showed that serum protein carbonyl contents were significantly increased ($p<0.001$) in diabetic patients as compared to normal human subjects. The average carbonyl contents (± SD) of three independent assays of diabetes serum proteins and normal human serum proteins were 3.51 ± 0.15 and 1.95 ± 0.14 nmoles/mg protein of the serum sample respectively (Fig. 104). Further, *in vitro* incubation with TQ (30 µM), TL (30 µM) and EU (0.6 µM) resulted in significant ($p<0.001$) decrease in the level of carbonyl group content in diabetic patients. The amount of protein bound carbonyl groups in diabetic serum samples treated with TQ, TL and EU was found to be 2.89 ± 0.15, 2.41 ± 0.11 and 2.05 ± 0.16 nmoles/mg of protein respectively. The treatment of EU was more effective than TL. TQ was found to be least effective (Table 12).

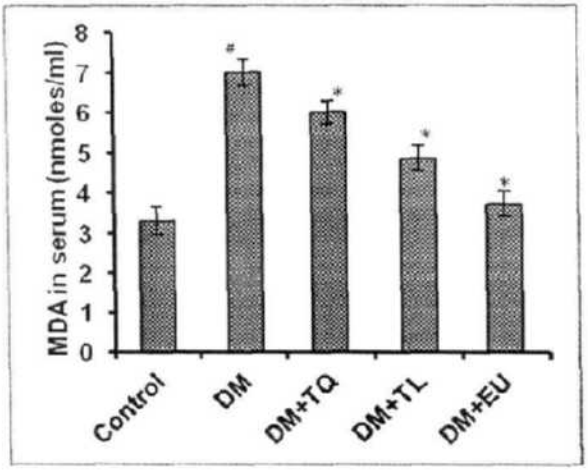

Fig. 103: MDA levels in serum of healthy (Control) and diabetic patients (DM) and in diabetic patient's serum treated with thymoquinone (DM+TQ), thymol (DM+TL) and eugenol (DM+EU). Data are expressed as Mean ± SD, n=40.

*p<0.001 as compared with DM.

#p<0.001 as compared with Healthy.

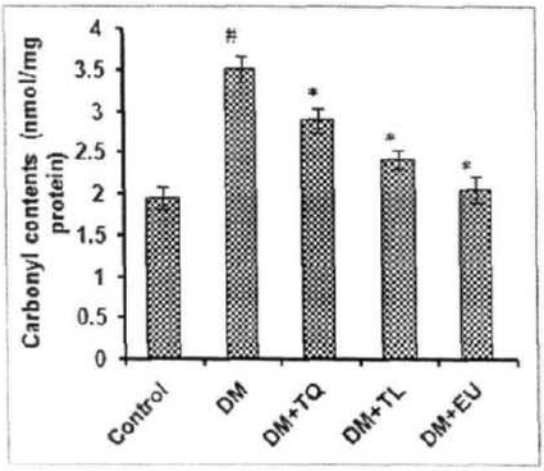

Fig. 104: Protein carbonyl level in serum of healthy (Control) and diabetic patients (DM) and in diabetic patient's serum treated with thymoquinone (DM+TQ), thymol (DM+TL) and eugenol (DM+EU). Data are expressed as Mean ± SD, n=40.

*p<0.001 as compared with DM.

#p<0.001 as compared with Healthy.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP assay was used for the determination of total antioxidants in serum samples of healthy and diabetic patients as well as in TQ (30 μM), TL (30 μM) and EU (0.6 μM) treated diabetic serum samples. As evident from Fig.105, FRAP value decreases significantly ($p<0.001$) in diabetic patients as compared to control. The average FRAP value (± SD) of three independent assays of diabetic serum and normal human serum were 719.5 ± 78.8 and 1270.1 ± 92.9 μM respectively. Furthermore, when the diabetic samples were treated with TQ (30 μM), TL (30 μM) and EU (0.6 μM), significant ($p<0.001$) increase in FRAP value was observed as compared to the diabetic samples without any treatment. The increment in FRAP value after treatment of diabetic samples with TQ, TL and EU was found to be 11%, 22.4% and 37.2% respectively (Table 12). The effect of *in vitro* incubation of diabetic patient's sera with EU is more pronounced than TL and TQ.

Estimation of reduced glutathione level (GSH)

Reduced glutathione is an important antioxidant in cellular system. Table 12 summarizes the glutathione level in healthy and type 2 diabetic patients. The present study reports a decline in reduced GSH content in serum of type 2 diabetic patients (13.7 ± 1.09 μM) as compared to healthy controls (25.7 ± 1.26 μM). In concordance to FRAP results in diabetic serum, phytochemical treatment ameliorates reduced GSH level significantly ($p<0.001$). Addition of EU (0.6 μM) induce highest increment in reduced glutathione level (23.2 ± 1.09 μM) in comparison to treatment of 30 μM TL and TQ in diabetic patients serum (Fig. 106).

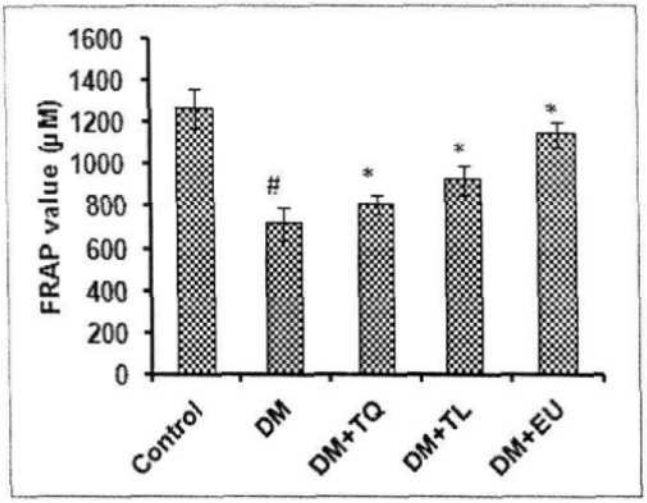

Fig. 105: FRAP value in serum of healthy (Control) and diabetic patients (DM) and in diabetic patient's serum treated with thymoquinone (DM+TQ), thymol (DM+TL) and eugenol (DM+EU). Data are expressed as Mean ± SD, n=40.

*$p<0.001$ as compared with DM.

#$p<0.001$ as compared with Healthy.

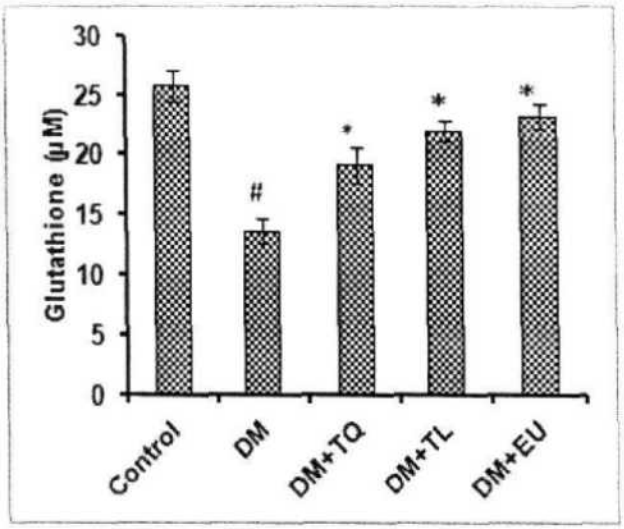

Fig. 106: Effect of TQ-2 (DM+TQ), TL-2 (DM+TL) and EU-2 (DM+EU) treatment on reduced glutathione level in type 2 diabetic patients (DM). Glutathione level in healthy individuals served as control. Each point represents the Mean ± SD (n=40) of three independent assays.

*$p<0.001$ as compared with DM.

#$p<0.001$ as compared with Healthy.

Effect of TQ, TL and EU on electrophoretic pattern of HSA in the sera of diabetic patients

One of the manifestations of diabetes is glycation of proteins. So, we observed the effect of phytochemicals, viz. TQ, TL and EU on crosslinking and/or fragmentation inhibition of HSA. Effect was observed on sera incubated with glucose and compared with sera incubated in same conditions in absence of glucose. As albumin is the major protein of human serum, hence incubated serum samples on SDS-PAGE analysis showed band of ~66 kD. Fig. 107(a) shows SDS-PAGE profile of diabetic patient 1 (D1). Serum profile of diabetic patient showed broadening of band towards high and low molecular weight, protein fragmentation and/or cross-linking but the sera incubated with TQ, TL and EU exhibited inhibition of diffusion of band and aggregate formation. It was further approved by gel densitometric analysis on SDS-PAGE of diabetic patient 1 (D1) (Fig. 107b). Amongst the three phytochemicals, EU at the 0.6 μM (Fig. 107a, lane 6) concentration could inhibit protein cross-linking and/or fragmentation better than 30 μM of TQ (Fi and TL. As observed in Fig. 108, diabetic patient's sera incubated with glucose shows increased cross-linking and diffusion of band due to fragmentation, but in the presence of phytochemicals glucose incubated sera exhibited decreased fragmentation and cross-linking. Again, EU at 0.6 μM was found to inhibit protein aggregates formation and cross-linking better than 30 μM of TQ and TL. Similar case was observed for other 9 patients with EU being most effective in inhibiting HSA glycation in diabetics (Fig. 109-126).

Table 12: Levels of protein carbonyl, MDA and FRAP in serum of healthy (Control), type 2 diabetic patients (DM) and diabetic serum samples treated with thymoquinone 30 µM (DM+TQ), thymol 30 µM (DM+TL) and eugenol 0.6 µM (DM+EU).

	CONTROL	**DM**	**DM + TQ**	**DM + TL**	**DM + EU**
Protein carbonyl (nmoles/mg protein)	1.96 ± 0.14	3.51 ± 0.16$^{\#}$ (+44.3%)^{a}	2.89 ± 0.15* (-17.74%)^{b}	2.41 ± 0.11* (-31.2%)^{b}	2.05 ± 0.16* (-41.7%)^{b}
MDA (nmoles/ml)	3.31 ± 0.34	7.01 ± 0.34$^{\#}$ (+52.8%)^{a}	6.02 ± 0.28* (-14.2%)^{b}	4.87 ± 0.32* (-30.5%)^{b}	3.73 ± 0.32* (-46.8%)^{b}
FRAP (µM)	1270.1 ± 92.9	719.5 ± 78.8$^{\#}$ (-43.3%)^{a}	808.2 ± 47.9 (+11%)^{b}	927.0 ± 68.3* (+22.4%)^{b}	1146.2 ± 59.5* (+37.2%)^{b}
GSH (µM)	25.7 ± 1.26	13.7 ± 1.09$^{\#}$ (-46.7%)^{a}	19.1 ± 1.48* (+28.3%)^{b}	22.0 ± 0.89* (+37.7%)^{b}	23.2 ± 1.09* (+40.9)^{b}

Results were expressed as Mean ± SD (n=40) of three independent assays.

$^{*}p<0.001$ as compared with DM.

$^{\#}p<0.001$ as compared with control.

a = % Change from control.

b = % Change from diabetic serum samples.

Diabetic patient (D1) without glucose

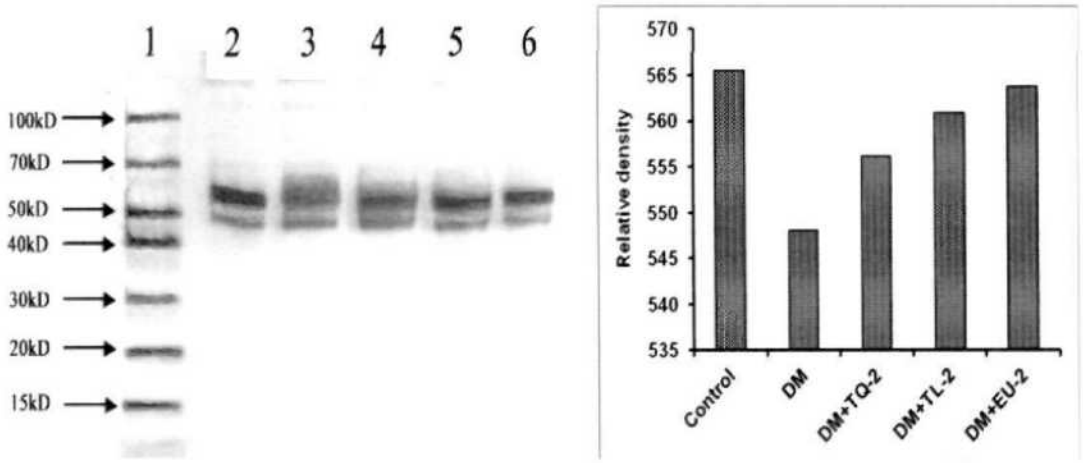

Fig.107: (a) SDS-PAGE of diabetic patient's serum (D1, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days; (b) Densitometric analysis of the same gel.

Lane 1: Molecular weight Marker in kD

Lane 2: Serum of Healthy subjects (10 µg protein)

Lane 3: D1 (10 µg protein)

Lane 4: D1 + TQ (30 µM)

Lane 5: D1 + TL (30 µM)

Lane 6: D1 + EU (0.6 µM)

Diabetic patient (D1) with glucose

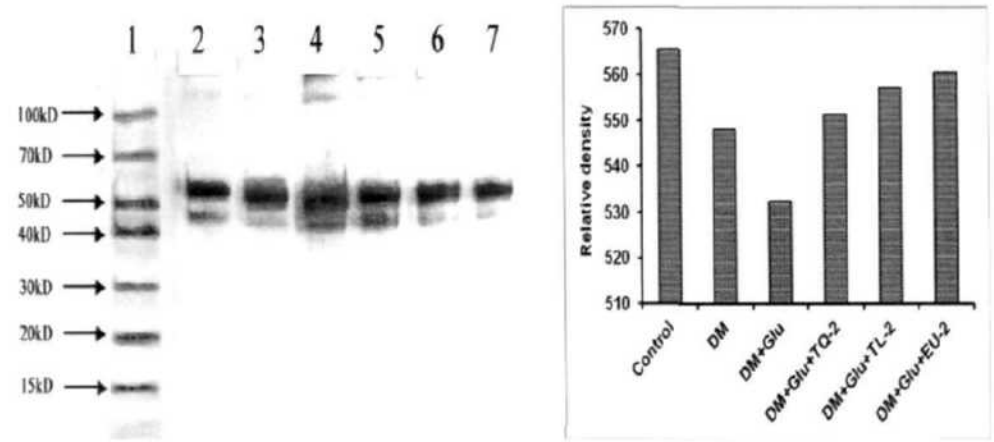

Fig.108: (a) SDS-PAGE of diabetic patient's serum (D1, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days; (b) Densitometric analysis of the same gel.

Lane 1: Molecular weight Marker in kD
Lane 2: Serum of Healthy subjects (10 µg protein)
Lane 3: D1 (10 µg protein)
Lane 4: D1 + Glucose (20 mM)
Lane 5: D1 + Glucose + TQ (30 µM)
Lane 6: D1 + Glucose + TL (30 µM)
Lane 7: D1 + Glucose + EU (0.6 µM)

Diabetic patient (D2) without glucose

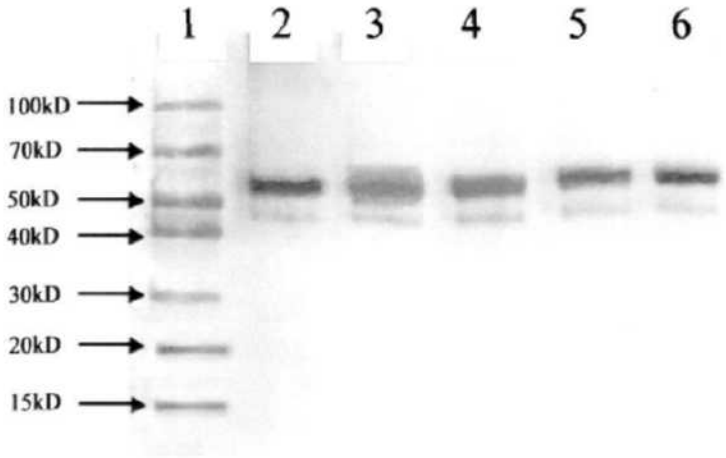

Fig.109: SDS-PAGE of diabetic patient's serum (D2, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig107.

Diabetic patient (D2) with glucose

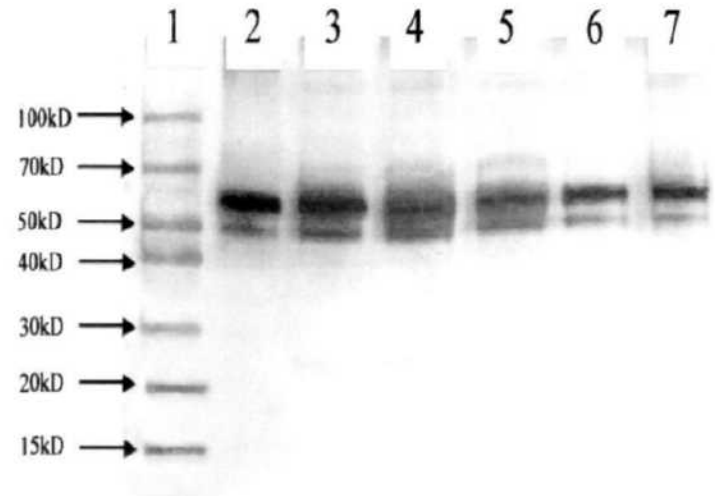

Fig.110: SDS-PAGE of diabetic patient's serum (D2, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig 108.

Diabetic patient (D3) without glucose

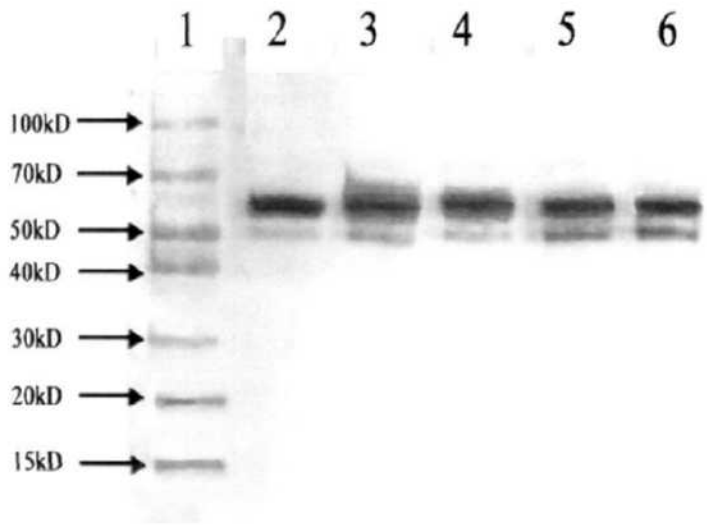

Fig.111: SDS-PAGE of diabetic patient's serum (D3, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig107.

Diabetic patient (D3) with glucose

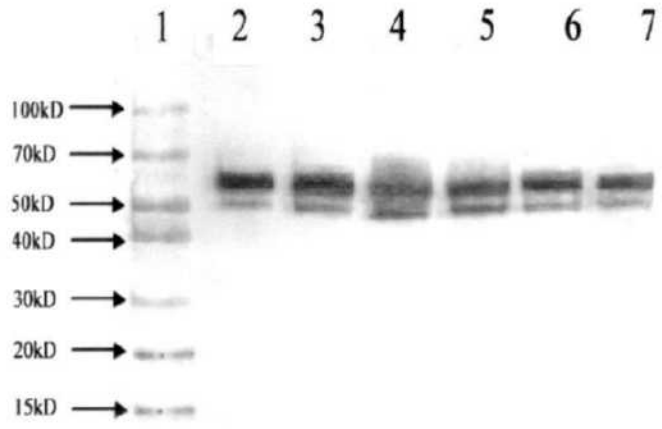

Fig 112: SDS-PAGE of diabetic patient's serum (D3, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig108.

Diabetic patient (D4) without glucose

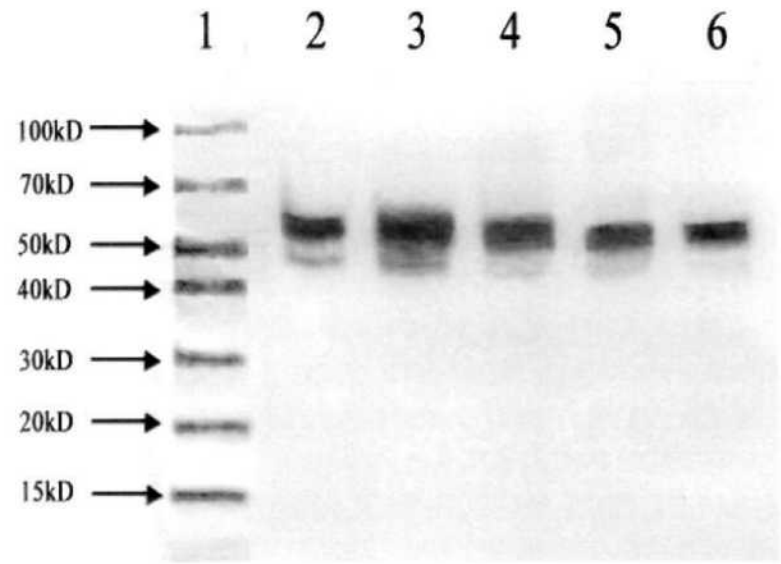

Fig.113: SDS-PAGE of diabetic patient's serum (D4, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D4) with glucose

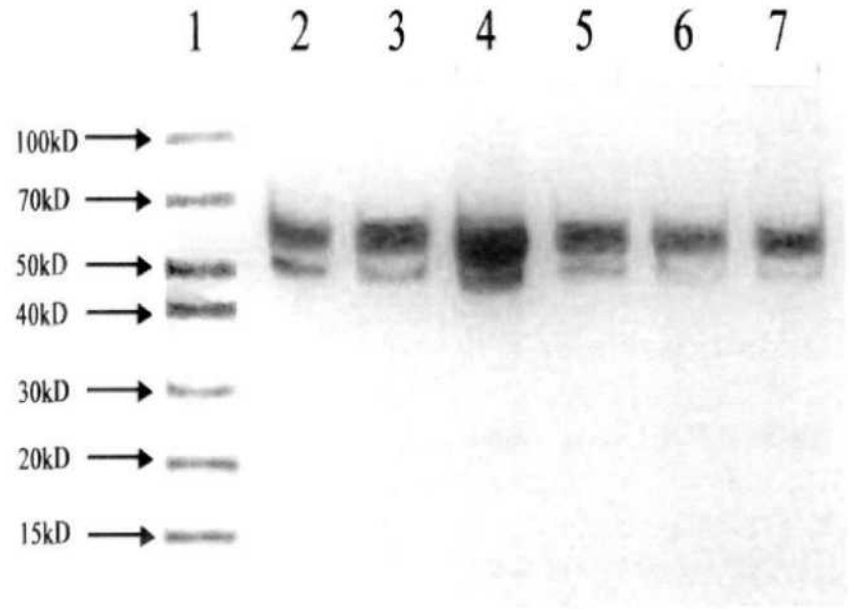

Fig.114: SDS-PAGE of diabetic patient's serum (D1, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D5) without glucose

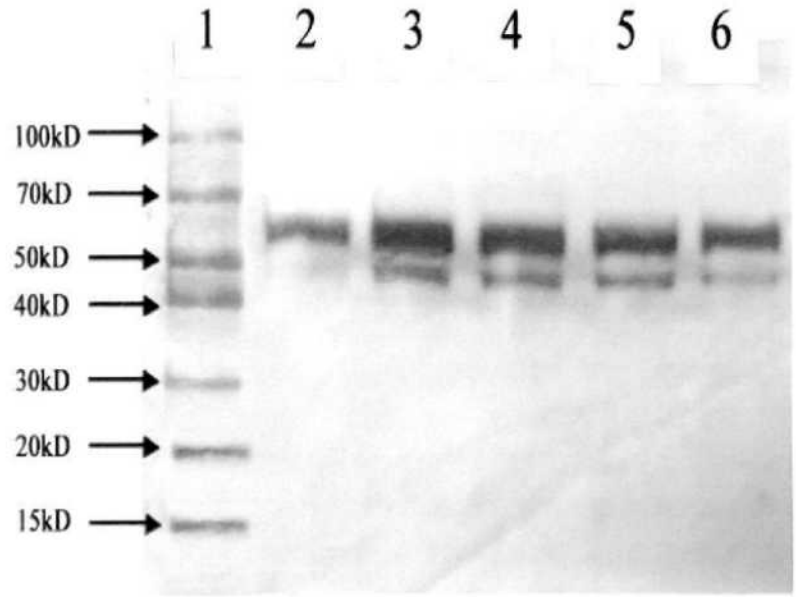

Fig.115: SDS-PAGE of diabetic patient's serum (D5, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D5) with glucose

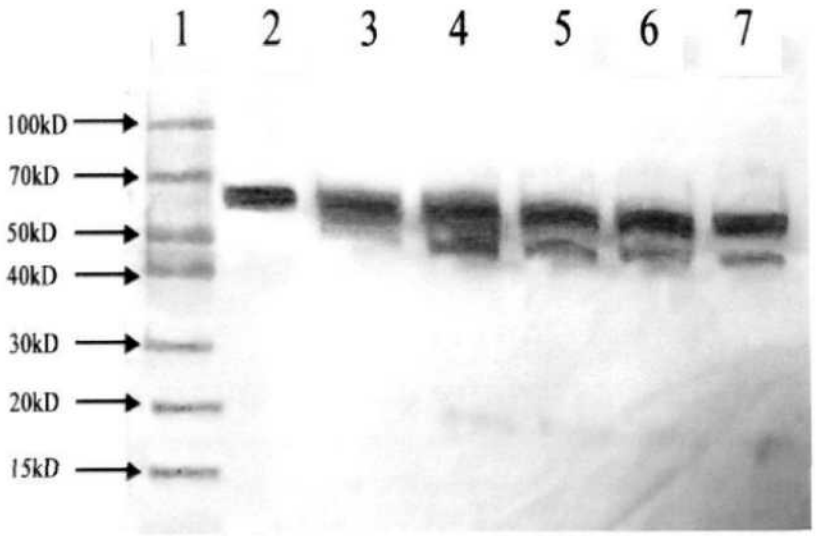

Fig116: SDS-PAGE of diabetic patient's serum (D5, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D6) without glucose

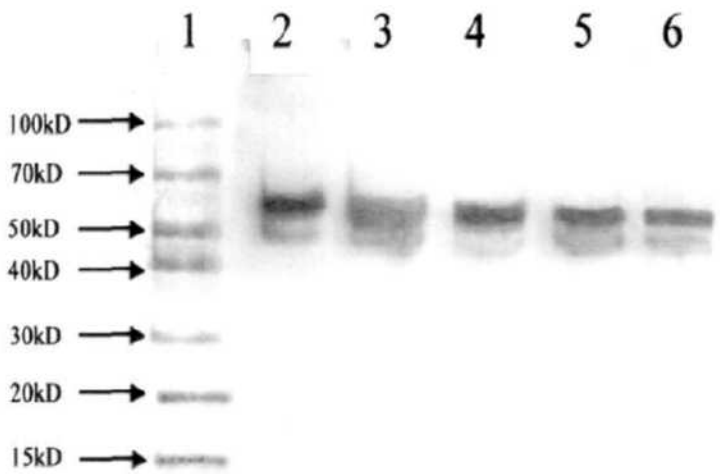

Fig.117: SDS-PAGE of diabetic patient's serum (D6, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D6) with glucose

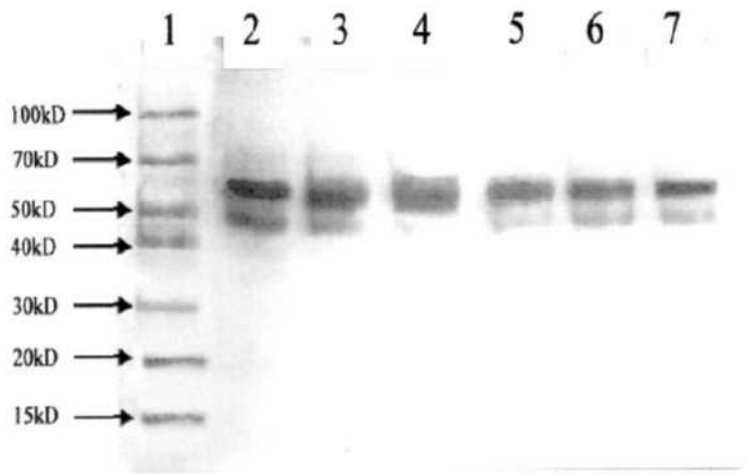

Fig. 118: SDS-PAGE of diabetic patient's serum (D6, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D7) without glucose

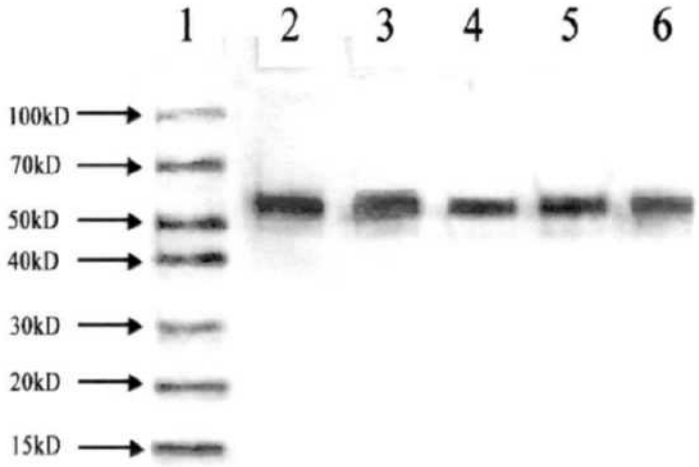

Fig.119: SDS-PAGE of diabetic patient's serum (D7, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D7) with glucose

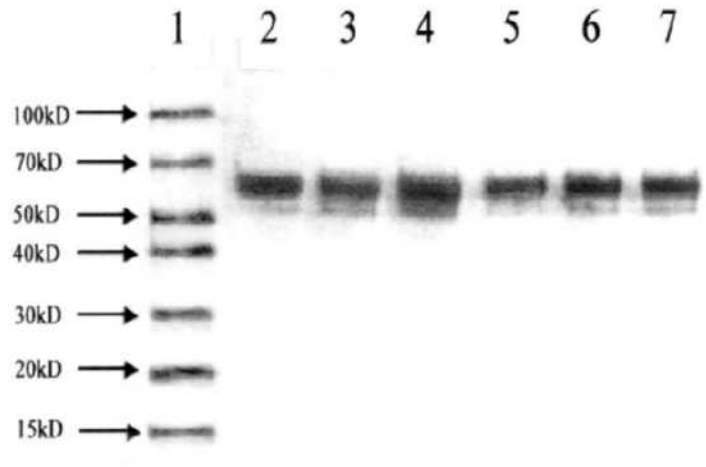

Fig. 120: SDS-PAGE of diabetic patient's serum (D7, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D8) without glucose

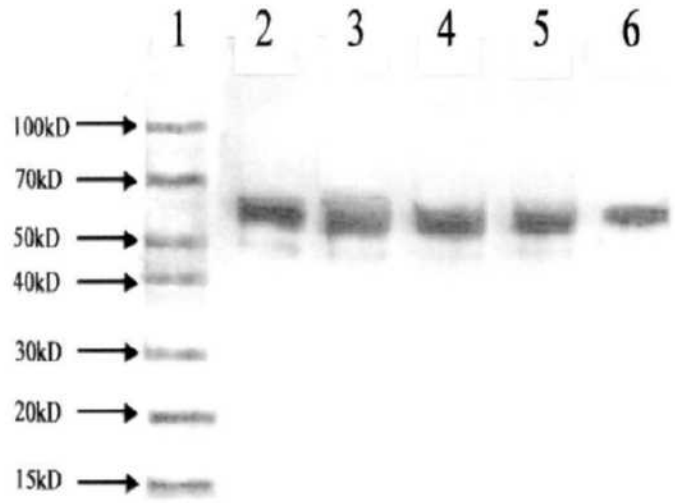

Fig.121: SDS-PAGE of diabetic patient's serum (D8, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D8) with glucose

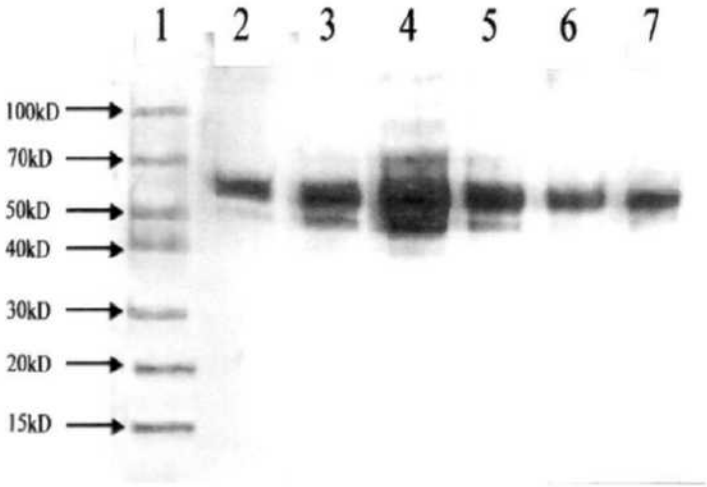

Fig.122: SDS-PAGE of diabetic patient's serum (D8, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D9) without glucose

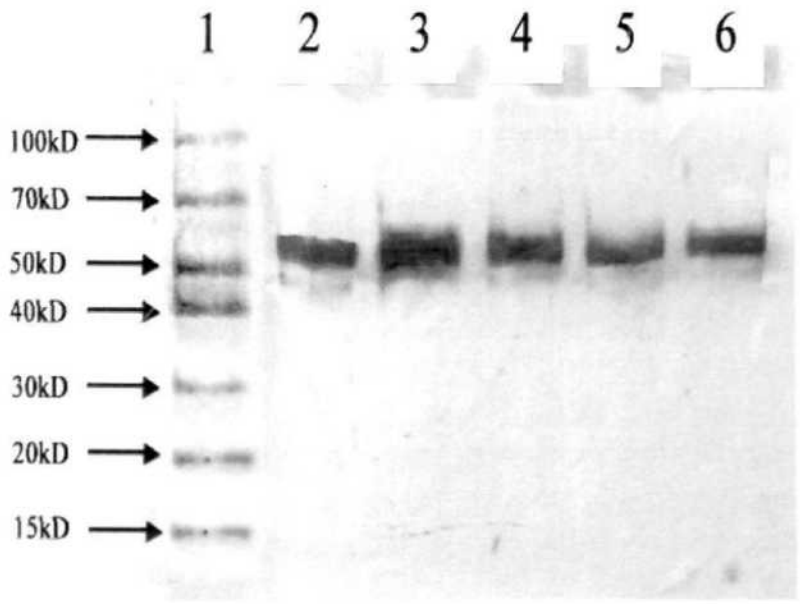

Fig.123: SDS-PAGE of diabetic patient's serum (D9, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D9) with glucose

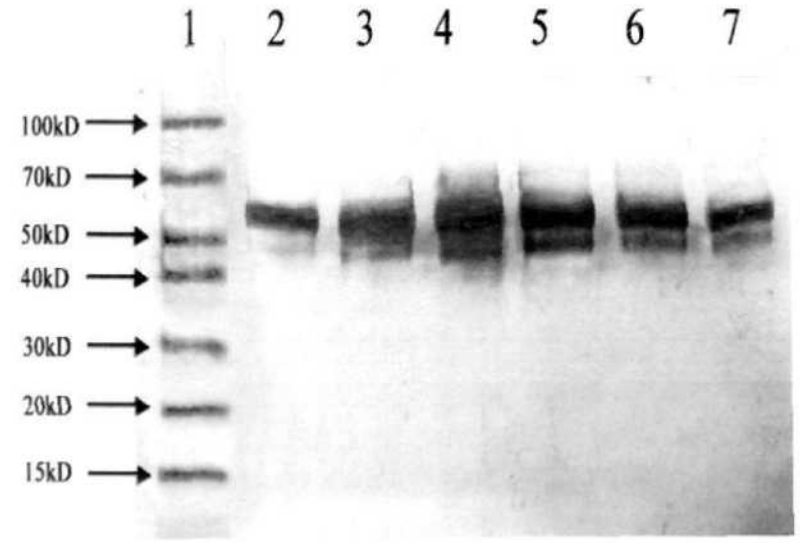

Fig. 124: SDS-PAGE of diabetic patient's serum (D9, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Diabetic patient (D10) without glucose

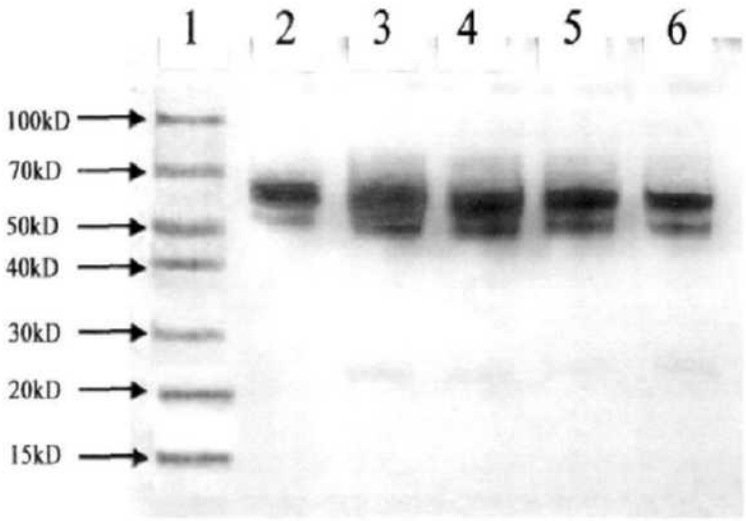

Fig.125: SDS-PAGE of diabetic patient's serum (D10, 10 µg protein) incubated with TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 107.

Diabetic patient (D10) with glucose

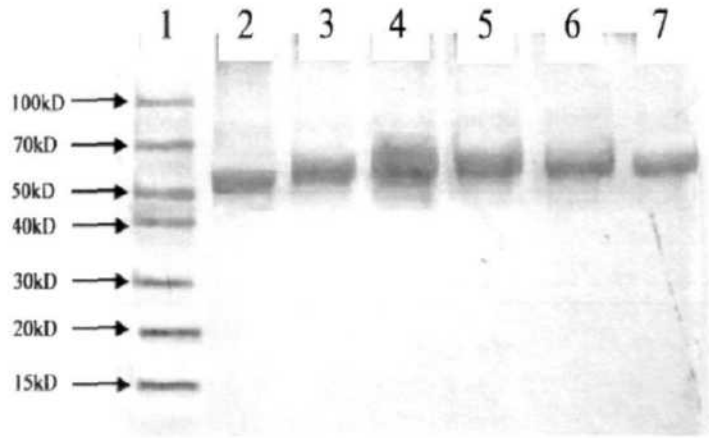

Fig. 126: SDS-PAGE of diabetic patient's serum (D10, 10 µg protein) incubated with 20 mM glucose in presence of TQ, TL or EU in 20 mM phosphate buffer, pH 7.4 at 37°C for 21 days. Remaining captions are same as in fig. 108.

Discussion

In general phenolics share the same chemical structure and composed of an aromatic hydroxyl nucleus (Karaman *et al.*, 2010). Majority of plant based phenolic compounds attracted a great deal of scientific interest because of their health promoting effects (Hollman and Katan, 1999). Plant phenolics generally act as primary antioxidants or free radical terminators. Interestingly, plant polyphenols are multifunctional in the sense that they can act as reducing agents, hydrogen atom donors and singlet oxygen scavengers. Certain polyphenols are effective as antioxidants chelating transition metal ions, which may otherwise induce Fenton-type oxidation reactions in their free states (Rice-Evans *et al.*, 1996).

Thymoquinone (TQ) (2-isopropyl-5-methyl-1,4-benzoquinone), is the main bioactive component of the volatile oil of *N. sativa* (Fig. 11). TQ is pharmacologically active benzoquinone and possesses antioxidant properties (Al-Shabanah *et al.*, 1998). It has also been used as anti-inflammatory and antineoplastic medicines for more than 2000 years (Trang *et al.*, 1993; Hosseinzadeh and Parvardeh, 2004). *Thymol* (TL) (2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, isomeric with carvacrol, found in oil of thyme. TL can be used for the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria (Didry *et al.*, 1994; Kohlert *et al.*, 2002). *Eugenol* (EU) (4-allyl-2-methoxyphenol) is a methoxyphenol compound having a short hydrocarbon chain in its structure (Gulcin, 2011). EU has been used as spice because of its strong odor and as a dental antiseptic because of its detergent-like effect (Tai *et al.*, 2002). It is also used for local anesthesia (Brodin and Roed, 1984) and as antistress (Sen *et al.*, 1992).

The present study focuses on antioxidant potential of TQ, TL and EU. Besides there have also been analyzed for their activities such as inhibition of AAPH induced RBC hemolysis; ferric ion-reducing power (Fe^{3+}-Fe^{2+} transformation); ferric reducing antioxidant potential (FRAP); 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging and metal chelating activities. These multiple methods are recommended to measure antioxidant properties of pharmacologic material that better reflect their potential protective effect.

The main mechanism responsible for antioxidants activity was considered to be the scavenging of free radicals, although other mechanisms may also be involved (Nenadis, 2003). DPPH is a free radical and has been widely used to test the free radical scavenging activity of various antioxidants (Shimoji *et al.*, 2002). Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radicals becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant the absorption strength decreases, and the resulting decolorization is stiochiometric with respect to the number of electrons captured (Blois, 1958). Our results showed that all phytochemicals presented a strong concentration dependent anti-free radical activity (Fig. 16). The scavenging ability against DPPH radical was high even at low concentrations, especially for EU. However, none of the phytochemicals were as effective DPPH scavengers as the positive control gallic acid (EC_{50} = 2.87 ± 0.40 µg/ml). These results evidenced that antiradical activities are in the order of TQ<TL<EU indicating that TQ, TL and EU neutralize the $DPPH^{\bullet}$ radical either by donation of electron or phenolic hydrogen to form a stable DPPH-H and a phenoxyl radical molecule (Gulcin, 2011). Radical scavenging capacity of each phytochemical might be correlated to their differential structure that favor phenolic hydrogen donation and the stability of the resulting phenoxyl radicals. In addition extension of the conjugation to the carbon chain is a molecular feature of EU which could participate by resonating structures leading to the stabilization of the phenoxyl radical (Nenadis, 2003). Conjugation seemed to enhance the antioxidant and radical scavenging activity of EU. It was reported that EU reduces 2 or more DPPH radicals despite the availability of only 1 hydrogen atom from a hydroxyl group, and different hypothesis have been proposed to explain the antiradical efficiencies of different monophenolic compounds (Mastelic *et al.*, 2008). Considering the absence of phenolic group in the TQ, its higher EC_{50} value was expected. Several other reports also showed effective scavenging activity of EU and TQ (Ito *et al.*, 2005; Khalife and Lupidi, 2007). In many studies scavenging effect of plant extracts having thymol as main component was shown (Nikolova, 2012).

Reducing power reflects the electron donating capacity of bioactive compounds and is associated with their antioxidant activity. In our study, the reducing power of three phytochemicals can be ranked in the order of TQ<TL<EU (Fig. 17). Interestingly, reducing power of EU exceeds that of ascorbic acid (AA) which was used as standard antioxidant. Ito *et al.,* (2005) also showed strong reducing power of EU. The reducing power might be due to electron/ hydrogen donating ability of bioactive compounds and may serve as a significant indicator of its potential antioxidant activity (Shimada, 1992).

FRAP assay provided the absolute antioxidant values of phytochemicals. The antioxidant properties of phytochemicals were ranked as TQ<TL<EU. This may be explained on the basis of their ferric ion reducing ability and electron donor properties for neutralizing free radicals by forming stable products. In our study, the trend of reducing abilities for three phytochemicals was similar to the result of scavenging effect on DPPH radical. EU exhibited the best effect in all test methods as EU with lowest EC_{50} exhibited high FRAP value (Fig. 18). During current study, TQ, TL and EU have been reported to have significant potential to chelate metal ions which was elucidated by its property to scavenge DPPH. Metal chelation activity of EU is higher than TQ and TL but marginally lower than AA (Fig. 19).

Antioxidant capacity is defined as the ability of a compound to inhibit oxidative degradation such as lipid peroxidation (Roginsky, 2005). Lipid peroxidation can be defined as oxidative deterioration of lipids containing several carbon-carbon double bonds. Excessive oxidative damage to cellular membranes contributes to the initiation and progression of numerous degenerative diseases, including certain types of cancer and diabetes (Pryor, 2000; Young and Woodside, 2001). Red blood cells are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals. Lipid oxidation of human red blood cell membrane mediated by AAPH induces membrane damage and subsequent hemolysis (Miki *et al.,* 1987; Zhu *et al.,* 2004). The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly relate with and quench peroxide radicals to terminate the peroxidation chain reactions (Soares *et al.,*

1997). Antioxidant intercepts the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl group, thereby forming a stable end product that does not initiate or propagate further oxidation of lipid (Soares *et al.*, 1997). Thus, *in vitro* oxidative hemolysis of human erythrocytes was used here as a model to study the free radical-induced damage of biological membranes and the protective effect of TQ, TL and EU in the process. In the present study, all phytochemicals inhibited AAPH (an azo peroxyl radical initiator) mediated human RBC hemolysis in a concentration-(Fig. 20) and time-(Fig. 21) dependent manner. When AAPH was added to the suspension of erythrocytes, hemolysis induction was time-dependent until 3 hour. The hemolysis is lagged, indicating that endogenous antioxidants in the erythrocytes, namely glutathione, tocopherol, ascorbate and enzymes such as catalase and superoxide dismutase, can efficiently quench radicals to protect them against free radical induced hemolysis, as described previously (Zou *et al.*, 2001). When production of peroxyl radicals overcomes the numerous antioxidant barriers of defense, an "oxidative stress" condition will develop, inducing oxidative damage on erythrocyte constituents, namely on membrane and haemoglobin, which may lead ultimately to hemolysis (Cimen, 2008). TQ, TL, EU and AA showed significant inhibition upto 86.22%, 86.69%, 89.18 and 90.72% at the concentrations of 100, 87.5, 21.875 and 50 µg/ml respectively. At a concentration of 25 µg/ml (after 3 hours), TQ inhibited RBC hemolysis by 6.78% which is 82%, 38% and 80% less than L-ascorbic acid, TL and EU respectively at a similar concentration. Since, it is known that polyphenolics enhance red blood cell resistance to oxidative stress both *in vitro* and *in vivo* (Youdim *et al.*, 2000). So, TL and EU having phenolic group in addition to nucleophilic benzene ring in all three phytochemicals may quench the chain propagating peroxyl radicals in the aquous phase to stop the peroxidation, hence inhibiting hemolysis more profoundly. This result is in accordance with the DPPH assay since EC_{50} of TQ was found to be much higher than that of TL and EU.

Among three phytochemicals, EU had the most powerful antioxidant and radical scavenging activity followed by TL and TQ which is related to their structure. Free radicals are the chemical species having at least one unpaired electron in the outermost shell and need electrons to complete their valence shell. Hence free radicals are

electrophile entities and show attraction towards nucleophilic molecules. So the compounds having maximum electron density shows the maximum antioxidant activity. Benzene ring is sufficiently nucleophilic in nature and attracts electrophilic groups. Furthermore, by analyzing the chemical structure of TQ, TL and EU we can note that all three are aromatic phytochemicals having nucleophilic benzene ring. Therefore, all three phytochemicals attract and scavenge free radicals. TQ has weak electron donating groups contributing to less electron density to the benzene ring. However, electron density is found to be higher in TL and EU as compared TQ because of presence of hydroxyl group attached to benzene ring. Oxygen of hydroxyl group donates its lone pair of electrons to benzene ring, thus increasing the electron density. Among EU and TL, EU has two electron donating groups viz. methoxy group (—OCH_3) and allyl group (—CH_2—CH=CH_2) at ortho- and para- position (more reactive sites as compared to meta- position) respectively while TL has weak electron donating groups (methyl and isopropyl) at ortho- and meta- position respectively (Fig. 12 and 13). Electron donating groups present at ortho- and para- are more reactive as compare to the meta- position. Hence it can be concluded that EU having maximum electron density showed best antioxidant activity among the three phytochemicals. Our results also indicate that TQ, TL and EU suppressed radical induced oxidative hemolysis of erythrocytes *in vitro*. To the best of our knowledge, this is the first report concerning the antihemolytic activity of these phytochemicals. The work herein indicates a high potential of application for these phytochemicals in food and drug products, with remarkable benefits for human health.

Non-enzymatic glycation and oxidation of some important biomacromolecules are crucial in the pathogenesis of several diseases such as diabetes (Jakus, 2003; Schmitt *et al.*, 2005). For example, hyperglycemia plays an important role in the pathogenesis of diabetic complications by inducing protein glycation with the gradual build up of AGEs in body tissues (Cohen *et al.*, 2005). Protein glycation and the formation of AGEs are accompanied by increased free radical activity that further contributes toward the biomolecular damage (Ahmed *et al.*, 2005). The elevated levels of glycoxidation products in plasma and tissue proteins are generally accompanied with oxidative stress in diabetes

(Ahmed *et al.*, 2005). AGE formation is an inevitable process *in vivo* and can be accelerated under oxidative stress.

Being the most abundant protein in blood constituting about 40 mg/ml plasma, we use human serum albumin as model protein (Shaklai *et al.*, 1984). In normal individuals nearly 10% of the HSA is modified by glycation, while there is 2-3 fold greater modifications under hyperglycemic conditions such as diabetes (Guthrow *et al.*, 1979). The glycated albumin is therefore considered a useful marker for short term monitoring (2-7 weeks) of diabetic patients. In fact, level of the glycated albumin reflects the degree of hyperglycemia in diabetes (Nakajou *et al.*, 2003). The conformational perturbation generated on incubation of HSA with glucose was determined by highly sensitive physico-chemical techniques such as spectral analysis, circular dichroism spectropolarimetry, FTIR, formation of ketoamine, carbonyl group, free amino group and polyacrylamide gel electrophoresis.

It is well recognized that reaction of protein with glucose results in marked alterations of its conformation (Ahmed *et al.*, 2005). The UV-absorption spectra of glycated HSA incubated for 7-14 days showed substantial hyperchromicity (Fig. 22). This may be due to unfolding of protein aggregates that are formed in the early phase of glycation process. The exposure of buried chromophoric groups to the polar environment leads to subsequent increase in the UV intensity. The glycated HSA incubated for 21 and 28 days demonstrated hypochromicity (Fig. 22). Glycation may have caused masking of aromatic amino acids due to aggregation of the protein leading to subsequent decrease in UV intensity (Traverso *et al.*, 1997). There was no apparent shift in λmax in all the incubation conditions.

The intrinsic fluorophore tryptophan is an excellent parameter to monitor the tryptophan environment in the protein and is highly sensitive to the polarity of surrounding environment (Ge *et al.*, 2011). The time-dependent plot of the tryptophan fluorescence intensity represents changes in protein structure in presence of glucose as revealed by Fig. 23. Glycated HSA incubated for 7 and 14 days depicted an increase in the fluorescence intensity as compared to native HSA. This might be due to unfolding of the

aggregates leading to exposure of chromophoric groups to the polar environment. Glycated HSA incubated for 21 and 28 days showed a decrease in the fluorescence intensity as compared to the native HSA indicating modification of tryptophan micro-environmental and/or masking of these groups due to the formation of aggregates (Davies *et al.*, 1987; Shaklai *et al.*, 1984). A decrease in intrinsic protein fluorescence caused by tryptophan has been previously reported by Cussons *et al.*, (1997) for glucose-modified HSA. Glycated HSA also showed a blue shift of 5 nm indicating that hydrophobic groups might be slightly more buried as compared to native HSA.

Several investigators have shown that reaction of sugars and aldehyde with proteins leads to the formation of groups like pentosidine which are considered characteristic biomarker of glycation and accompanying autoxidation. These adduct show strong emission between 400-500 nm when excited at a wavelength of 370 nm (Schmidt *et al.*, 2001; Traverso *et al.*, 1997). With increase in time of incubation with glucose, there was consistent increase in the peak value of emission in range 400-500 nm when compared to control indicates the formation of glycation adducts and AGEs. As evident from the Fig. 24, AGEs formation was maximum between 14 days and 21 days but stopped off subsequently.

For further analysis of tertiary and secondary structural changes in native and glycated HSA, circular dichroism spectropolarimetry was carried out. It is useful for rapid assessment of secondary structures of proteins. In the spectral region of 200-250 nm, the CD signals of proteins are obtained mainly due to the amide chromophores of the proteins' peptide bonds (Hennessey *et al.*, 1981; Manavalan *et al.*, 1987). CD spectra also showed alterations in the helical behavior of HSA. Proteins rich in α-helix give negative bands near 208 and 222 nm whereas, proteins containing pure β-sheets display negative bands near 216 nm. The incubation of HSA with glucose appears to promote conformational change leading to instability in the secondary structure and decrease in percent helicity also. Incubation of HSA with glucose leads to a decline in the negative CD signal which started decreasing slightly with time in HSA samples incubated with glucose showed a gradual disappearance of the minima at 208 and 222 nm. After 28 days

there was disappearance of negative CD signal at 208 and 222 nm and appearance of minima at 217 nm occur which indicate the β-sheet structure (Fig. 25). The spectrum indicates α-helix to β-sheet transformation between 21 and 28 days. The α-helical content was determined for protein secondary structure analysis, and in the case of native HSA we found 64% α-helix which is in close agreement with the available literature value (Gull *et al.*, 2007; Liu *et al.*, 2005). However, upon glycation with glucose, the α-helical content of the protein decreased from 64% to 61% after 7 days. Further incubation of HSA with glucose up to 28 days showed marked decrease in α-helical content to 48% (Table 4). This signifies perturbation in the secondary structure of HSA upon glycation by glucose.

Far-UV CD results were further supported by FT-IR spectroscopy, a powerful method for investigating the secondary structure of proteins. It helped us locate the position of characteristic bands in native and glycated HSA. The infra-red spectrum of protein exhibits a number of amide bands which represent different vibrations of peptide moieties (Rahmelow and Hubner, 1996). The frequencies of bands due to the amide I, II and III vibrations are sensitive to the secondary structure of proteins. Amide I band is more sensitive to the change of protein secondary structure than amide II. The amide I peak position occurs in the 1600-1700 cm^{-1} region mainly associated with the C=O stretching vibration of the peptide linkage and corresponds mainly to α-helix. Amide II band position occurs from 1480-1575 cm^{-1} (C–N stretch coupled with N–H bending mode) (Liu *et al.*, 2003; Zheng *et al.*, 2010). Peaks observed in native HSA confirms the purity of HSA as per established standard (Li *et al.*, 2010) (Fig. 26a). The shifting of amide I and II bands indicate changes in intra-molecular bonding which arise from the impact on overall protein confirmation due to glycation. Peak observed at 1158.83 cm^{-1} in glycated HSA is suggestive of opening of the epoxy ring of HSA on interaction with glucose (Fig. 26b) (Li *et al.*, 2010; Shibata, 2011).

The reaction with NBT is a standard method for the detection of formed ketoamines which allowed characterizing the binding of glucose to the primary amino groups on the HSA molecules (Ahmed *et al.*, 2002). Ketoamines are early non-enzymatic glycation

adducts and are important precursors of AGEs and hydroxyl radicals. It was found that ketoamine generation in glycated HSA incubated for 7 days was significantly higher than its native form (Fig. 27). It is well recognized that carbonyl groups are introduced in protein as a consequence of glycation. The generation of carbonyl groups serves as a marker of protein glycoxidation (Baynes and Thorpe, 1999; Beal, 2002). Ketoamines were converted to protein bound carbonyl compounds via a protein enediol reaction (Baynes, 1991). Incubation of HSA with glucose resulted in a time-dependent introduction of carbonyl groups in the molecule up to 21 days (Fig. 28). There was no marked increase in carbonyl groups for further incubation up to 28 days. Number of free amino groups of proteins irreversibly modified due to non-enzymatic glycation (Traverso *et al.*, 1997). As the time increases there was a decrease in free amino group of glycated HSA as shown by percent decrease in free amino groups as compared to native HSA (Fig. 29).

Another parameter used for the study of protein glycation was the formation of protein cross-links. Electrophoretic migration allows the evaluation of such modifications in a protein (Chesne *et al.*, 2006). The SDS-PAGE of glycated HSA showed a decrease in the band intensity (as indicated by gel densitometric analysis) as well as broadening of the band towards high and low molecular weights (Fig. 30a), which can be attributed to extensive inter- and intramolecular cross-linking due to glycation, resulting in the formation of aggregates (Bouma *et al.*, 2003; Rosca *et al.*, 2005). Interestingly, samples incubated with glucose migrated as highly diffused bands with the increase in incubation time up to 28 days and showed the presence of aggregates and fragmentation. The changes were more obvious at 21 and 28 days of incubation. This could also be seen in the densitometric analysis of gels showing a decrease in density due to protein fragmentation (Fig. 30b).

On the basis of above observations we can infer that incubation with glucose for up to 28 days resulted in a time-dependent modification of HSA. The changes were more prominent in HSA incubated with glucose for 21 days. Thus, prolonged exposure of HSA to glucose exerts greater deleterious effects on its structure and formation of aggregates.

Preliminary data in the present study indicates formation of glycated HSA involving covalent linkage of free amino groups to glucose. It was shown earlier that while amino groups are primary targets, the extent of glycation does not directly depend on the number of glycation-prone residues but on the solvent accessible surface area of such residue (GhoshMoulick *et al.*, 2007).

The accumulation of various protein glycation products in living organisms lead to functional modifications of tissue protein. Many studies have shown a significant role for glycation in the pathogenesis of diabetic complications (Brownlee, 1995; Basta *et al.*, 2004). Thus the discovery and design of glycation inhibitors offer a promising therapeutic approach for its prevention (Rahbar and Figarola, 2003). Although, the synthetic glycation inhibitor aminoguanidine, attenuates the development of a range of diabetic complications, some toxicity problems have been encountered in clinical trials with this drug (Thornalley, 2003). For last decade, attention has been focused on preventing protein glycation by antioxidants from plant sources (Rudnicki *et al.*, 2007). Glycation and AGE-induced toxicity are also known to be associated with increased free radical production. Recent studies have demonstrated the benefits of using compounds with combined antioxidant and antiglycation properties (Nakagawa *et al.*, 2002; Ahmad and Ahmed, 2006). Such compounds not only prevent AGE formation but also reduce free radical-mediated toxicity.

There are several reports that various plant products show remarkable AGE inhibitory activity. These include resveratrol, a natural stilbenoid phenol present in grapes, curcumin found in turmeric, S-allyl cysteine a constituent of garlic, rutin found in tomatoes and many polyphenolic compounds in Zea mays. Green tea contains tannins having significant antiglycation properties (Mizutani *et al.*, 2000; Nakagawa *et al.*, 2002). It is well known that phenolic compounds are constituents of many plants (Rice-Evans, 1997). Some antioxidants also act as inhibitors of glycation (Urios *et al.*, 2007). The antioxidant properties of the phenolic compounds can be attributed to their potential as hydrogen- or electron-donating agents and metal ion chelating properties mediated considerably free radical scavenging properties (Rice-Evans *et al.*, 1996).

Most of the glycation inhibitory phytochemical constituents of plants have been reported to possess polyphenolic nature (Rudnickira *et al.*, 2007). The present study explores and compares the effect of co-incubation of TQ, TL and EU on glucose mediated glycation of model protein HSA up to 28 days. Besides, structural changes interaction of HSA with glucose cause marked alterations in its conformation (Ahmed, 2005). The resulting covalent cross-linking may induce stress in the molecule, triggering the overall conformational changes as revealed by hyperchromicity and gain of intrinsic fluorescence up to 14 days (Table 5, 6) (Khan *et al.*, 2011). This may be explained by protein unfolding in the initial phase of glycation reaction that led to exposure of buried groups to the external milieu (Traverso *et al.*, 1997). After 14 days of incubation there was hypochromicity and loss of intrinsic fluorescence that may be due to protein folding and aggregation that cause exposure of aromatic amino groups. Interestingly, incubation of HSA with glucose in presence of varying concentrations of TQ, TL and EU was found to alleviate conformational changes in the protein (Fig. 31-64). The protective property of these phytochemicals could be explained on the premise that these phytochemicals may act as quenchers of glucose/carbonyl compounds thereby inhibiting their interaction with protein leading to prevention of glycation-induced conformational changes in HSA.

AGEs cause various types of protein modifications resulting in structural and functional impairments such as intra- and inter cross-linking, absorption and fluorescence at specific wavelength (Bucala and Cerami, 1992; Fu *et al.*, 1994). It has been proposed that oxidation plays a crucial role in the formation of fluorescence and the molecular bridge which are characteristic feature of AGEs (Fu *et al.*, 1994). The AGEs complications are a direct result of protein alteration which results in irreversible tissue damage. Thus, inhibition of the formation of AGE is believed to play a role in the prevention of diabetes related complications. Therefore, the identification of antiglycation compounds is considerably an attracting interest. The known AGEs inhibitors act either as quenchers of dicarbonyl intermediates, as metal ion chelators or as antioxidants (Verbeke *et al.*, 2000; Rahbar and Figarola, 2003). Some herbal extracts and natural products have proven to be somewhat effective for inhibiting AGE formations (Kim and Kim, 2003). Based on the fluorescence properties, we studied the influence of TQ, TL and EU on the formation of

AGEs and employed AG, a well-known AGE inhibitor. Our results demonstrated that all three phytochemicals efficiently inhibited AGEs formation as observed by a reduction in the AGE-associated fluorescence (Table 7). Moreover, a strong inhibitory activity against the formation of AGEs was shown by EU followed by TL and TQ (Fig. 65-77). This could be explained by structure-activity relationship. Glucose reacts with amino groups of proteins forming ketoamines which ultimately leads to AGEs formation. Further, oxidation processes are also important in the formation of many AGEs (Fu *et al.*, 1994). There are two mechanisms through which oxidative processes take palce. The first involves autoxidation of glucose, leading to formation of ketoaldehydes, more reactive dicarbonyl compounds, which react with proteins to form highly reactive ketoamine, finally leading to AGEs formation. The second mechanism involves oxidative breakdown of amadori products to form highly reactive protein-enediols and protein dicarbonyls which can generate AGEs. Therefore, both the Amadori formation and glucose autoxidation may contribute to the development of diabetic complications via free radical-induced protein oxidation and promotion of AGEs formation (Takagi *et al.*, 1995). Recent studies have indicated that some radical species, including H_2O_2, $O_2^{\bullet-}$ and singlet oxygen participate in AGEs formation (Chace *et al.*, 1991; Yim *et al.*, 1995), and that antioxidant and radical scavengers inhibit these processes (Oya *et al.*, 1997). Since the formation of AGEs is facilitated under oxidative reactions, all the three phytochemicals used in the study inhibit their formation by decreasing the ROS formation or by trapping the ROS formed *in vitro* by autoxidation of glucose and/or oxidative degradation of Amadori products. This hypothesis is reinforced by the results of our antioxidant parameters which have shown that TQ, TL and EU have potent antioxidant and radical scavenging effect (TQ<TL<EU) which has been shown by reducing power, FRAP assay and DPPH scavenging assay, inhibition of generation of $O_2^{\bullet-}$ along with metal chelating properties. Moreover, the above hypothesis is also strengthened and well supported by the observation that these phytochemicals also suppress oxidative protein damage in terms of protein carbonyl formation which is believed to cause decrease in the glycoxidation processes. Further, the FTIR studies showed that phytochemical treatment causes shift in the peaks of amide I and II bands.

Interestingly, some new peaks were also observed (Fig. 78) confirming the formation of new bonds. This suggests the interaction of all three phytochemicals with either HSA or glucose thereby limiting their interaction.

Ketoamines are early non-enzymatic glycation adducts. It was shown earlier that while amino groups are primary targets, however, the extent of glycation does not directly depend on the number of glycation prone residues but on the solvent accessible surface area of such residues (GhoshMoulick *et al.*, 2007). The treatment of glycated HSA at 7 days of incubation with TQ, TL or EU showed slightly lower levels of ketoamine (Fig. 79, Table 8) as compared to untreated glycated HSA. Although the exact mechanism of inhibition of ketoamine formation is at present unknown, this could be explained by the possibilities of mechanisms such as quenching of glucose/carbonyl compounds, thereby limiting the reactivity towards the amino groups of protein or phytochemicals may also bind with HSA. Binding to glucose or protein would inhibit ketoamine production and subsequent AGE formation.

It is well recognized that glycation reactions contribute to protein damage. Further, autoxidation of glucose as well as oxidative degradation of Amadori product enhance the chemical modifications of proteins by ROS (Wolff and Dean, 1987; Bucala, 1992). In fact, carbonyl groups are introduced in the proteins as a consequence of glycation (Beal, 2002). In addition, oxidation of protein is also accompanied by the formation of carbonyl protein. Thus protein carbonyl content is considered to be a reliable measure of glycoxidation (Telci *et al.*, 2000). Regarding the presence of free radicals and oxidative steps in the glycoxidation process compounds with antioxidant activities have been tested in order to reduce or stop glycoxidation. In this study, we examine antioxidant viz., TQ, TL and EU mediated protection of HSA against glycoxidation. We found that exposure of HSA to glucose could enhance the protein oxidation levels in terms of protein carbonyl formation, while all three phytochemicals inhibits glucose-induced glycoxidative damages to protein as indicated by reduction in protein carbonyl formation (Fig. 80-84, Table 9). This clearly suggest that three phytochemicals have potential to alleviate oxidative stress by inhibiting oxidative processes may be effective in preventing

glycoxidative protein damage. Potency of the protein carbonyl inhibitory activity was in the order of TQ<TL<EU suggesting the relationship between carbonyl inhibitory activity, free radical scavenging activity with the chemical structure of phytochemicals.

At an early stage of Maillard reaction, both free as well as ε-amino groups of lysine residues of HSA reacts with the carbonyl groups of reducing sugars. Moreover, reactive carbonyl species can react with lysine and arginine residues forming specific AGEs (Chace *et al.*, 1991). Some AGEs are formed on the same lysine residue that was initially modified by glucose, whereas others can form intra- and intermolecular covalent cross-links with unmodified lysine or arginine residues, thus propagating initial modifications (Gunusen *et al.*, 2010). The measurement of free lysine residues indicates that in the glycated HSA samples, there were significantly less free lysine residues than in the control. The presence of phytochemicals inhibits the modification of lysine residues indicating that in their presence glucose cannot react with the lysine residues of HSA. This may be due to quenching of glucose or carbonyl compounds. Moreover, an increase in free lysine residues in presence of these phytochemicals confirms that none of them chemically react with amino groups in the lysine residues of HSA (Fig. 85-89, Table 10). Moreover, the phytochemicals inhibit AGEs formation thereby inhibiting intra- and intermolecular cross-links with unmodified lysine or arginine residues that eventually ensued in increase in free lysine residues.

It is well known that ROS are produced at many steps during sequential glycation cascade. Schiff's base, Amadori product and AGEs etc. generated during this process led to the formation of free radicals. Several reports indicate the production of radicals and highly reactive oxidants from glycated proteins under physiological conditions (Wolff *et al.*, 1991). It has also been reported that glycated proteins generate fifty times more superoxide radicals *in vitro* than unmodified proteins and cause a two-fold increase in peroxidation of lipid vesicles (Mullarkey *et al.*, 1990). Superoxide radical is generated when the Amadori product takes on an enaminol structure. These free radicals in turn will induce further oxidative protein degradation (Wolff and Dean, 1987). Recent studies have indicated that some radical species including H_2O_2, superoxide anion radicals and singlet

oxygen participates in AGEs formation (Chace *et al.*, 1991) while antioxidant and radical scavengers inhibit these processes (Yokozawa and Nakagawa, 2004). To evaluate whether TQ, TL and EU could affect superoxide radical formation during glycation, we employed cytochrome c reduction assay on day 7 of incubation. There was a significant increase in the amounts of superoxide radical in glycated HSA. Addition of TQ, TL and EU inhibited the increase in superoxide radical (Fig. 91). The observed effect on inhibition of superoxide radical generation in phytochemical treated glycated HSA could be explained on the basis of their antioxidative or free radical scavenging properties (TQ<TL<EU). EU has a methoxyphenolic structure that plays an important role in antioxidant functions (Fujisawa *et al.*, 2002) while TL lacks methoxy group. TL and EU being the phenolic compounds exhibited considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents and metal chelating properties (Rice-Evans *et al.*, 1996). However, TQ showed least superoxide anion radical scavenging activity due to the presence of less reactive and weak electron donating groups attached to ortho- and meta- position.

Reactive carbonyl species formed during glycation can react with protein forming specific AGEs (Chace *et al.*, 1991). These AGEs can form intra- and intermolecular covalent crosslinks leading to formation of high molecular aggregates (Liggins and Furth, 1996; Ahmad *et al.*, 2007). Moreover, glycation is also accompanied by autoxidation of glucose and Amadori products to produce free radicals capable of protein fragmentation (Wolff and Dean, 1987; Bucala and Cerami, 1992). HSA was glycated by glucose in presence of different concentrations of TQ, TL and EU with subsequent analysis of glycation-derived crosslinking and/or protein fragmentation using SDS-PAGE (Fig. 92-102). Phytochemicals restricted glycation-induced protein crosslinking and/or protein fragmentation to some extent. The photographed gels were also analyzed through densitometry and showed the increase in density in presence of phytochemicals confirming the above finding (Table 11). These phytochemicals probably act by preventing autoxidative glycation and glycoxidative reactions. This could also be attributed to their ability to prevent, multiple changes associated with the accumulation of AGEs, i.e., reduction in carbonyl stress and prevention of oxidative stress etc.

In conclusion, our findings herein showed that all three phytochemicals are powerful antioxidant and have potent inhibitory effect on protein glycation and glycoxidation thereby preventing glucose mediated HSA modification *in vitro*. It seems these phytochemicals may act at multiple steps involved in glycation process and AGE formation. Interestingly, antiglycating activities were relevant and directly related to their free radical scavenging capability TQ<TL<EU. Inhibition of free radical produced from protein glycation and subsequent inhibition of protein modification is one of the mechanisms of glycation prevention (Farrar *et al.*, 2007). EU-2 was found to be the best inhibitor amongst various phytochemicals at all the time points. The high antioxidative efficacy of all three phytochemicals is more apparent from the fact that in comparison to AG that acts at magnitude of millimolar, the three phytochemicals are effective at micromolar concentration.

Diabetes mellitus is a metabolic disorder which is characterized by hyperglycaemia and insufficiency of secretion or action of endogenous insulin. Protein glycation and fragmentation of AGEs are accompanied by increased free radical activity (Ahmed *et al.*, 2005). The elevated levels of glycoxidation products in plasma are greatly accompanied with oxidative stress in diabetes (Ahmed *et al.*, 2005). Increased free radical production and reduced antioxidant defense responses, encountered in the diabetic state may give rise to oxidative stress (Baynes and Thorpe, 1999; Ceriello *et al.*, 2000). MDA, measured as TBARS, is the most commonly used marker of lipid peroxidation (Nishigaki *et al.*, 1981; Altomare *et al.*, 1992). Carbonyl groups are generated on protein side chains especially of arginine, lysine, proline and threonine in the oxidation reaction. Since these moieties are chemically stable and formed relatively early hence are exploited for detection. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cysteine, Histidine, and Lysine residues, with aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation (Dalle-Donne, 2003).

Antioxidants play a vital role as preventive factors in the pathogenesis of diabetes (Kaneto *et al.*, 1999). Therefore, much attention has been focused on the use of natural antioxidants/phytochemicals to inhibit lipid peroxidation, or to protect the damage from

free radicals. The present study, provide further evidence for the strong antioxidant effect of TQ, TL and EU in serum from diabetic patients.

In the present study, samples from diabetic patients had been found to possess significantly higher ($p<0.001$) serum levels of MDA than healthy subjects (Fig. 103). Several other reports also showed that due to oxidative stress, lipid peroxidation increases in diabetic patients (Nacitarhan *et al.*, 1995). It might be due to increase in the levels of oxygen free radicals which suggest their increased production. This increased level of MDA could be because of increased free radical production via glycated protein autoxidation of glucose as well as oxidative degradation of Amadori products in diabetes mellitus (Wolff and Dean, 1987; Bucala and Cerami, 1992). ROS formed causes peroxidative breakdown of phospholipid fatty acids and accumulation of MDA (Jain *et al.*, 1989). However, on incubation with TQ, TL or EU the MDA level decreases up to certain extent in diabetic patients (Table 12). This could be attributed to their ability of scavenging ROS.

We estimated the protein carbonyl content as a marker of protein glycoxidation. Our results showed that there is significant increase in the carbonyl group content in diabetic patients as compared to the control groups (Fig. 104, Table 12). Increased ROS production in diabetes via glycated protein autoxidation of glucose and oxidative degradation of Amadori product enhance the chemical modification of protein (Wolff and Dean, 1987; Bucala and Cerami, 1992). Carbonyl groups are introduced in the proteins as a consequence of glycation and oxidation (Beal, 2002). Our result is in concordance with earlier reports. Odetti *et al.*, (1999) showed higher carbonyl content in type 2 diabetic patients and that impaired glycemic control is connected to protein oxidation. On incubation with TQ, TL and EU the protein carbonyl content decreased up to 17.7%, 31.2% and 41.7% in diabetic patient's serum respectively. However, protein carbonyl level in diabetic samples on treatment with EU reaches very close to the normal value as shown in Fig. 104. Thus these phytochemicals showed antioxidative property by scavenging ROS, thereby protecting protein from free radical damage due to increase in oxidative stress.

On the other hand, total antioxidant capacity reflected by FRAP and GSH value was found to be significantly reduced in type 2 diabetic patients as compared to healthy individuals. The observed decrease in the level of FRAP and GSH in diabetic patients are an indicator of oxidative stress condition. It can also be considered as compensatory mechanism for minimizing the overwhelming stress and restoring the normal condition. Protein carbonyl content showed a direct relationship with MDA level in diabetic patients whereas FRAP assay and GSH level showed negative relation with protein carbonyl and MDA which was also observed in many reports (Nacitarhan *et al.*, 1995). Kuppusamy *et al.*, (2005) observed a significantly lower FRAP level in type 2 diabetic patients in both Malay and Indian ethnics. Colak *et al.*, (2005) documented a similar decrease in type 2 diabetic patients with cardiovascular complications. According to Kim *et al.*, (2007) study, the plasma levels of reduced GSH were significantly diminished in diabetic patients as compared to controls.

Treatment with TQ, TL and EU resulted in significant increment in FRAP value in type 2 diabetic patients (Fig. 105). TQ incubation showed lowest increment in the FRAP value (11%) while TL showed 22.4% increase in type 2 diabetic serum samples which is in concordance with their antioxidant and antiglycating properties. As compared to TQ and TL, EU showed excellent effect. Similarly, the level of GSH increased in type 2 diabetic patients on TQ, TL and EU treatment (Fig. 106). It showed that these phytochemicals protect cell components from damage due to oxidative stress and lower the level of free radicals in the body by their high reducing power and scavenging ability.

Glycation of proteins has been shown to result in protein degradation and/or crosslinking (Singh, 2001; Rosca *et al.*, 2005). Further, diabetic serum samples were also subjected to SDS-PAGE and showed the decrease in intensity and broadening of band towards high and low molecular weight. This could be attributed to fragmentation, extensive inter and intra-molecular cross-linking due to glycation, resulting in the formation of aggregates. Interestingly, diabetic serum samples incubated with glucose migrated as highly diffuse bands and showed fragmentation and the presence of aggregates. Further, decrease in density of bands in diabetic samples as compared to healthy subjects as showed in gel

densitometry (Fig. 107b, 108b), proved protein cross-linking and/or fragmentation due to glycation confirming the above finding.

However, electrophoretic pattern of diabetic serum samples treated with phytochemical in presence and absence of glucose are shown in Fig. 107-126. Our finding herein showed the reduced formation of glycation adduct in presence of TQ, TL or EU. The results suggested the potential of these phytochemicals in reducing the glycation-induced protein damage in the order, viz. TQ<TL<EU by reduction in carbonyl stress and prevention of oxidative stress. Inhibition of free radical generation derived from protein glycation and subsequent inhibition of protein modification is one of the mechanisms of prevention of glycation (Farrar *et al.*, 2007). These results are in accordance with earlier data pertaining to antioxidant and antiglycation parameters and provide further evidence for their strong antioxidant and antiglycation effect.

In order to analyze the antioxidant and antiglycative effect *in vivo*, it is important to evaluate the bioavailability of these phytochemicals. Plasma concentrations of EU were reported to be about 5 μM in human 2 hours after the administration of 150 mg EU (Fischer and Dengler, 1990). And the concentration of EU that can prevent glycation of HSA effectively as demonstrated in this study was 0.6 μM, indicating that EU can act as a physiological antioxidant and antiglycating agent *in vivo*. Although, the exact mechanism by which TQ, TL and EU function in the human body is not clear, however, the data of present study may have important therapeutic implications in view of increasing evidence that oxidative stress may be the underlying cause for most of the diabetic complications (Baynes and Thorpe, 1999) and the protection provided by these phytochemicals is a convincing reason for the possibility of using these phytochemicals in tackling of diabetic complications, as another mode of diabetes treatment not dependent on the control of blood glucose level. They may also help to reduce oxidative stress and concomitant free radical damage in diabetic patients. However, the *in vitro* results may not reflect the effects of these agents *in vivo*, as their antioxidative properties may decrease during cooking process as well as the liver-first pass effect, which invariably affect the content, activity and bioavailability of bioactive compounds. Hence, further investigation is needed to establish whether these phytochemicals attenuate glucose mediated protein modification *in vivo*.

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List of Publications

1. **Aman, S.**, and Moin, S. (2013). Antioxidant activity of thymoquinone and its protective effect against oxidative hemolysis. *International Journal of Scientific Research, 2(4)*, 28-30.
2. **Aman, S.**, Moin, S., Owais, M. and Siddiqui, M.U. (2013). Antioxidant activity of thymol: protective role in AAPH-induced hemolysis in diabetic erythrocytes. *International Journal of Pharmaceutical Science Invention, 2(3)*, 55-60.
3. Mahmood, T., Moin, S., Faizy, A.F., Naseem, S., and **Aman, S.** (2013). *Nigella sativa* as an antiglycating agent for Human Serum Albumin. *International Journal of Scientific Research, 2(4)*, 25-27.
4. Parasurampuria, P., Khan, A.A., Ashraf, M., **Aman, S.**, and Moin, S. (2013). Study of serum nitric oxide levels in senile cataract patients and in normal individuals. *International Journal of Scientific Research, 2(6)*, 417-419.

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Research Paper

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Antioxidant Activity of Thymoquinone and its Protective Effect Against Oxidative Hemolysis

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ABSTRACT

Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) play major role in various diseases like diabetes, cancer, neurodegenerative and immunodeficiency diseases etc. Detoxification of such reactive oxygen intermediates is an essential target, yet naturally occurring antioxidants are warranted for better management of diseases. In this study, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, reducing power and Ferric Reducing Antioxidant Power assay were used for evaluating the antioxidant activity. It was also tested that whether TQ in-vitro protects red blood cells from (AAPH)-induced hemolysis in diabetic and healthy subjects. It scavenges upto 78.13%. TQ demonstrated significant ferric reducing ability at very low concentrations in healthy and diabetic plasma samples. Furthermore, TQ was able to inhibit RBC hemolysis by 86.22% and 71.04% in normal and diabetic patients respectively. Our findings reveal that thymoquinone possess excellent antioxidant properties and could serve as a free radical scavenger, and this justifies its uses in alternative medicines.

1.INTRODUCTION

ROS are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function, or excessive quantities, the state called oxidative stress. Intracellular production of those oxygen intermediates threatens the integrity of various biomolecules including proteins [1], lipids as well as lipoproteins and DNA. ROS are the most lethal byproducts of metabolism that mediate many diseases including cancer, diabetes, immunodeficiency diseases and ageing [2,3]. There should be a balance between antioxidants and prooxidants to maintain the body 's optimal physiological conditions. Synthetic antioxidants, such as BHT and BHA have recently been suspected to cause negative health effects and therefore their applications have been restricted [4].

Many studies have demonstrated a correlation between the oxidative stress defense and the antioxidant properties of phytoconstituents [5,6]. Phenolic phytochemicals found in singnificant quantities in vegetables, fruits, spices and seeds. They have been regarded as possible antioxidants. Their roles in food industry and in chemoprevention of diseases resulting due to oxidative stress have become an area of active research [7,8].

TQ (2-isopropyl-5-methyl-1,4-benzoquinone), is the main bioactive component of the volatile oil of the black seed or black cumin (Nigella sativa, Ranunculaceae family). It has been used as antioxidant, anti-inflammatory and antineoplastic medicines for more than 2000 years [9,10]. Generally Nigella sativa seeds contain more than 30% fixed oil and 0.40% to 0.45% volatile oil [11]. TQ represents 18.4 to 24% of the N. sativa volatile oil [12]. The pharmacological investigations of the seed extracts reveal a broad spectrum of activities including antidiabetic [13], neuroprotective [14], antiasthmatic [15], anti-inflammatory [16] and antimicrobial [17]. In this study, we investigated the antioxidant properties of TQ and its effect on AAPH-induced hemolysis in erythrocytes obtained from diabetic patients and healthy subjects.

1.MATERIALS AND METHODS

Thymoquinone (TQ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich, USA. Rests of the general chemicals used were of analytical grade bought from Sisco (India), HiMedia (India) and Qualigens (India).

2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Activity

The free radical scavenging activity of TQ and standard reference compound i.e, Gallic acid was analyzed by the DPPH assay as described by Sanchez-Moreno et al. (1998)[18] with minor modification. In this assay, 1 ml of varying concentrations of TQ (0.25-2.0 mg/ml) dissolved in 1 ml of ethanol, were mixed with 1 ml of ethanol solution of DPPH (0.2 mM). The mixture was vortexed and incubated for 30 min. The optical density of the solution was measured at 517 nm using Hitachi 2010 spectrophotometer. Gallic acid (µg/ml) has been used as a standard. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

Radical scavenging activity (%) = [(OD control – OD sample)/ OD control] X 100

Reducing Power

Total reducing power was determined as described by Zhu et al., (2002)[19] with some modifications. TQ (0.5-2.0 mg/ml) in 1 ml of ethanol were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3Fe(CN)6]; the mixture was then incubated at 50°C for 30 minute. 2.5 ml of trichloroacetic acid (10%) was then added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml FeCl3 (0.1%), and the absorbance was measured at 700 nm.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP asssay was carried out by the method of Benzie & Strain (1996)[20] with slight modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe3+ -TPTZ) to its ferrous (Fe2+ -TPTZ), intensive blue colored form in the presence of antioxidant. 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM FeCl3 were mixed in a ratio of 10:1:1 to be a working FRAP reagent. 100 µl of TQ was mixed with 3ml of FRAP reagent and incubated at 37°C for 30 min. The absorbance at 593 nm was monitored. All reagents were freshly prepared before used. Aqueous solution of known Fe (II) concentration was used for calibration (in a range of 100-1000 µmol/l).

AAPH-induced RBC hemolysis assay

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J. N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered saline (PBS), pH 7.4. During every wash, RBCs were centrifuged at 4000rpm for 10 min to obtain packed cell preparation [21]. The packed RBC was suspended in four volumes of PBS solution after the last wash. AAPH, a peroxyl radical initiator, was used for RBC hemolysis [21]. Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. 0.5 ml of the erythrocyte suspension was mixed with 0.5 ml of PBS solution containing varying amounts of TQ. 0.5 ml of 200 mM AAPH was added. The reaction mixture was shaken gently while being incubated at 37°C for 3 hr. After incubation, reaction mixture was diluted with eight volumes of PBS and centrifuged at 4000 rpm for 5 min. The Absorbance (A)

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Antioxidant activity of thymol: protective role in AAPH-induced hemolysis in diabetic erythrocytes

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ABSTRACT: *In the aerobic environment, reactive oxygen species are the most lethal by-products of metabolism that mediate many diseases including cancer, diabetes, immune deficiency diseases and ageing. Present study is aimed to assess the antioxidant activity of Thymol and its implication in diabetes in vitro. Thymol was evaluated by ferric reducing antioxidant power (FRAP) assay and measurement of its total reducing power, total phenolic content and 1,1-diphenyl 2-picrylhydrazyl (DPPH) scavenging activity. Furthermore, the extract in vitro was also checked if it protects red blood cells (RBCs) from 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis in the samples of diabetic patients and healthy subjects.*

Thymol demonstrated good ferric reducing ability and high reducing power that can be attributed to its higher amount of phenolic contents. Moreover, it scavenges upto a great extent. Thymol was able to inhibit RBC hemolysis by 86.69±2.34% and 72.04±2.1% in normal and diabetic patients respectively.

Our findings reveal that thymol possess antioxidant properties and could serve as a free radical scavenger, and this justifies its uses in folkloric medicines.

Keywords: *Thymol, Diabetes, Antioxidants and ROS.*

I. INTRODUCTION

In the aerobic environment, reactive oxygen species are the most lethal byproducts of metabolism that mediate many diseases including cancer, diabetes, immunodeficiency diseases and ageing. There should be a balance between antioxidants and prooxidants to maintain the body's optimal physiological conditions. Synthetic antioxidants, such as BHT and BHA have recently been suspected to cause negative health effects and therefore their application have been restricted [1].

Many studies have demonstrated a correlation between the oxidative stress defense and the antioxidant properties of phytoconstituents [2,3]. Phenolic phytochemicals found in singnificant quantities in vegetables, fruits, spices and seeds. They have been regarded as possible antioxidants. Their roles in food industry and in chemoprevention of diseases resulting due to oxidative stress have become an area of active research [4,5,6,7,8].

Thyme has been commonly used in foods mainly for the flavor, aroma and preservation and also in folk medicine since the ancient Greeks, Egyptians and Romans. Thymol, carvacrol and terpinine are major constituents of the oils of thyme [9,10]. Thymol can be used for the treatment of oral infectious diseases because of their inhibitory activity on oral bacteria [11,12].

In this study, we investigated the antioxidant properties of thymol and its effect on AAPH-induced hemolysis in erythrocytes obtained from diabetic patients and healthy subjects.

II. MATERIALS AND METHODS

2.1. Materials

Pure thymol, granule and white solid substance, from Sigma. 2,2- diphenyl-1-Picrylhydrazyl (DPPH) and 2,2-azobis (2-methyl propion-amidine) dihydrochloride (AAPH) from Aldrich.

2.2. Methods

2.2.1. Determination of Total Phenolic Content

Total phenolic content was determined by the method of [13] with slight modification. In each analysis, 1.58 ml of distilled water was pipetted into test tubes, followed by addition of 20 µl of standard solution, sample solution, or water. Vortex all tubes. Then 100 µl of Folin-Ciocalteau's (FC) reagent was added to each test tube, and the solutions were mixed again. After 30s and before 8 minute, 300µl of 20% sodium carbonate solution was added. The solutions were left at room temperature for 2 hour. Then the absorbance of the developed blue colour was determined at 765 nm. Gallic acid was used as a standard for the calibration curve and reported as gallic acid equivalents (mg) using the following equation based on the calibration curve:

$A = 0.0013x + 0.00409$ $R^2 = 0.99765$